Vol. 22, No. 1. April, 1959

THE JOURNAL OF APPLIED BACTERIOLOGY

(Formerly Proceedings of the Society for Applied Bacteriology)

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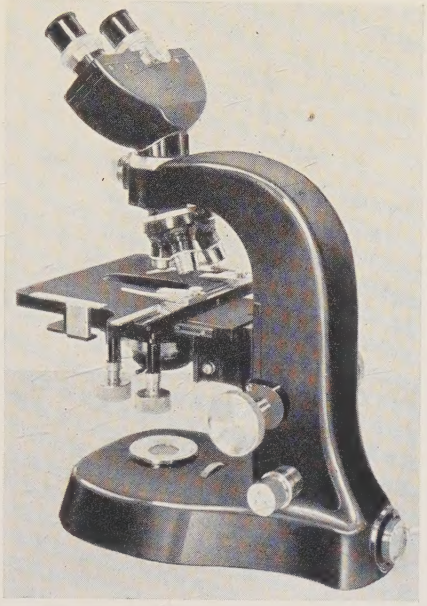

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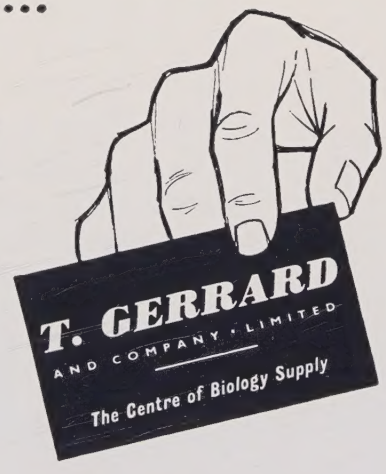

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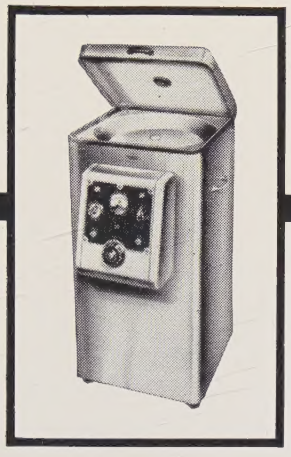

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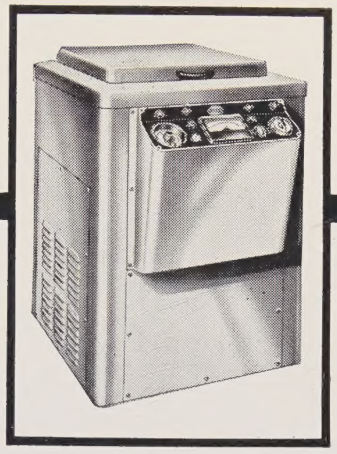

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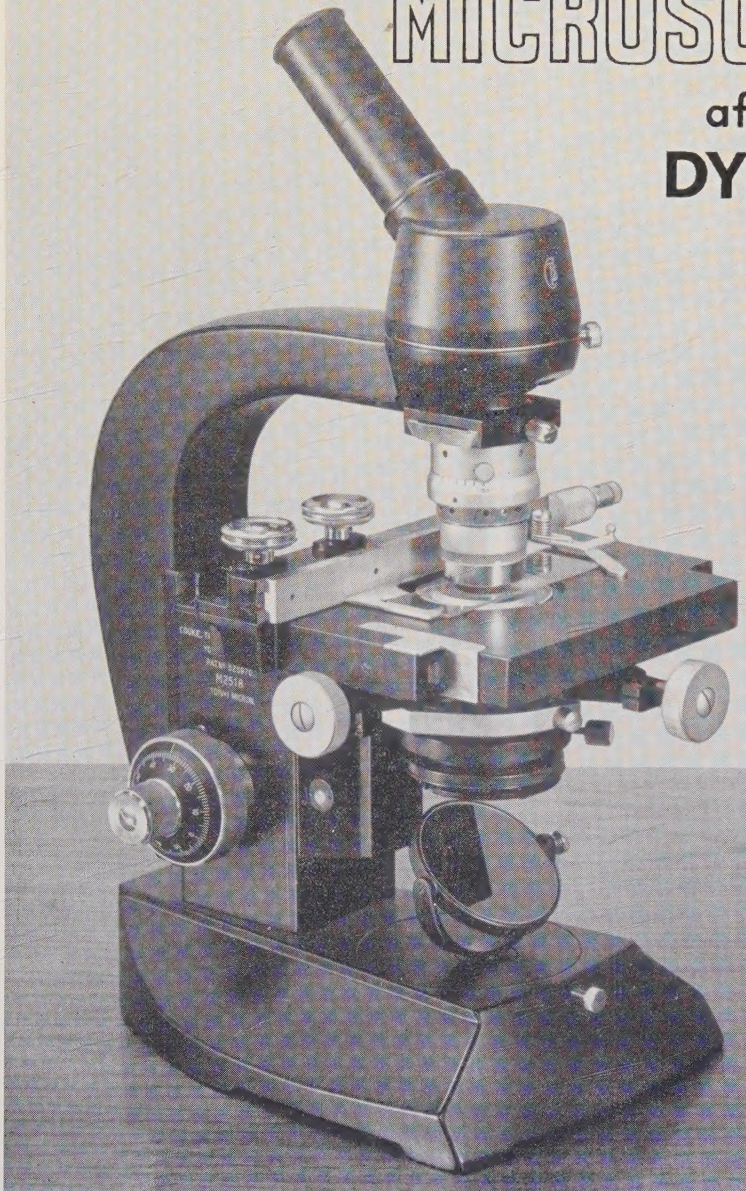

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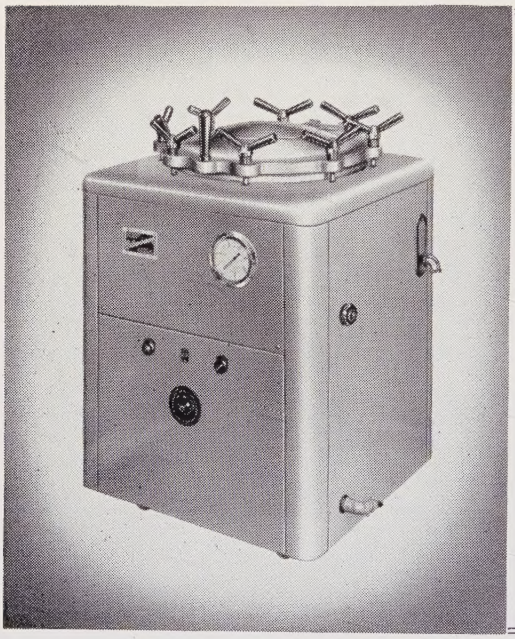

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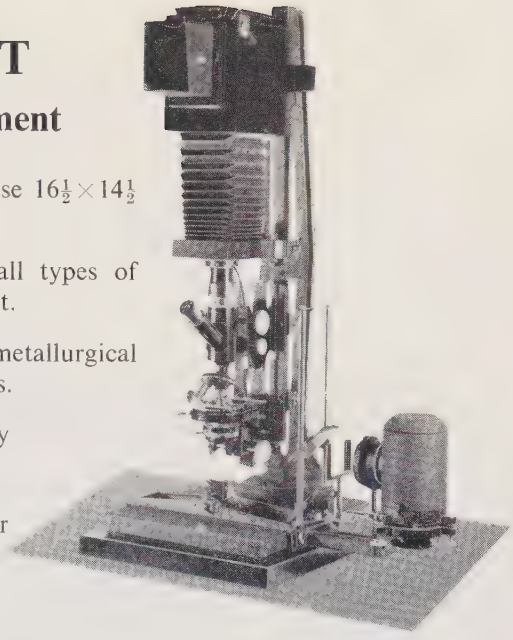

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CONTENTS OF THE JULY ISSUE

The Society in Retrospect and Prospect, by F. C. WHITE
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Modern Trends in the United Kingdom Dairy Industry
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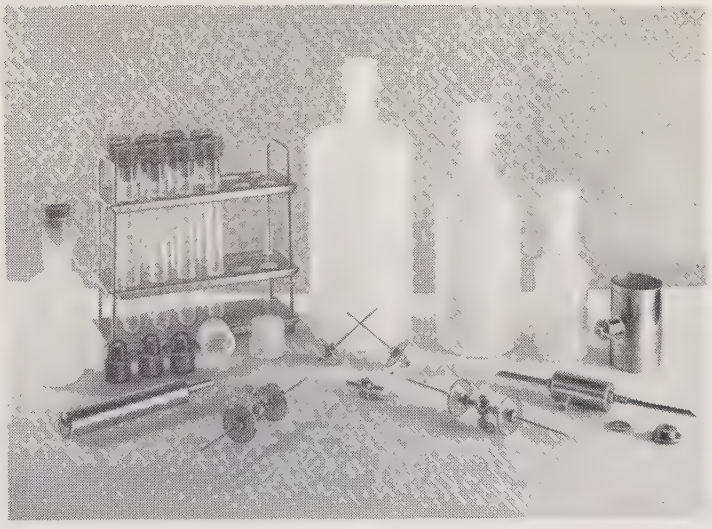

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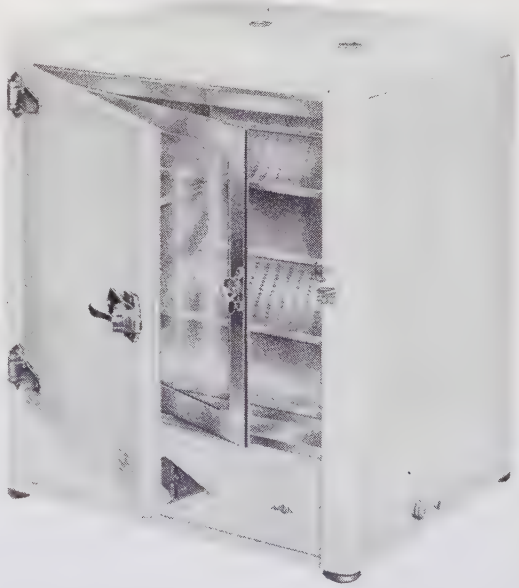

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Contents of Vol. 21, No. 1, August, 1959

Wilson, G. S. Faults and fallacies in microbiology. The Fourth Marjory Stephenson Memorial Lecture.
Watanabe, T. & Watanabe, M. Transduction of streptomycin resistance in *Salmonella typhimurium*.
Watanabe, T. & Watanabe, M. Transduction of streptomycin sensitivity into resistant mutants of *Salmonella typhimurium*.
Contois, D. E. Kinetics of bacterial growth: relationship between population density and specific growth rate of continuous cultures.
Ivanovics, G., Alföldi, L. & Nagy, E. Mode of action of megacin.
Smith, H. G. On the nature of the selective action of selenite broth.
Demain, A. L. & Hendlin, D. "Iron transport" compounds as growth stimulators for *Microbacterium* sp.
Band, R. N. Nutritional and related biological studies on the free-living soil amoeba, *Hartmannella rhysodes* Singh.
Kushner, D. J. & Lisson, T. A. Alkali resistance in a strain of *Bacillus cereus* pathogenic for the larch sawfly, *Pristiphora erichsonii*.
Williams, M. A. Chromatin patterns in *Spirillum anulus*.
Sheinin, R. The localization of the cell-bound penicillinase of *Bacillus cereus* in protoplasts.
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Briggs, S., Crawford, K., Abraham, E. P. & Gladstone, G. P. Further observations on the relationships between Gram-negative rods and staphylococci grown in the presence of penicillin.
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APPLIED BACTERIOLOGY

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OF
APPLIED BACTERIOLOGY

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Contents

No. 1 (*issued* 13 *November*, 1959)

PAGE

The Effect of Nisin on the Growth of Cells and Spores of *Clostridium welchii* in Gelatine. By J. E. EASTOE and JOAN E. LONG 1

The Inactivation of *Bacillus subtilis* Spores in Penicillin by Gamma Radiation. By E. C. HILL and G. O. PHILLIPS. (With one figure) 8

Mycological Synthesis of Fat from Whey. I. Preliminary Studies with Stationary Cultures. By P. WIX and M. WOODBINE 14

A Study of a Gram-Positive Coccus Responsible for Ropiness and Viscosity in Malt Wort. By J. W. HOPTON and A. N. HALL 23

Coli-Aerogenes Bacteria in Farm Water Supplies. By S. B. THOMAS, PHYLLIS M. HOBSON and R. G. DRUCE 32

The Bacterial Content of Farm Dairy Equipment. By D. G. GRIFFITHS and S. B. THOMAS 46

The Microbiological Examination of Butter. By R. G. DRUCE and S. B. THOMAS 52

Cold-Tolerant Fermentative Gram-Negative Organisms from Meat and Other Sources. By B. P. EDDY and A. G. KITCHELL 57

Some Observations on Bactericidal Paint Films. By E. J. MORRIS and H. M. DARLOW 64

The Sanitation of Fish Boxes. I. The Quantitative and Qualitative Bacteriology of Commercial Wooden Fish Boxes. By R. SPENCER 73

A Study of the Bacteria Associated with the Roots of Subterranean Clover and Wimmera Rye-Grass. By JOAN I. SPERBER and A. D. ROVIRA. (With two plates) 85

The Influence of Environmental Factors on the Microbicidal Effect of Ionising Radiations. By B. A. BRIDGES and T. HORNE. (With one figure) . . 96

Observations on the Isolation of Salmonellae from Selenite Broth. By H. G. SMITH. (With four figures) 116

The Effect of Freezing and Storage on the Bacterial Flora of Pasteurized Milk. By J. G. MURRAY and W. E. COEY 125

The Occurrence of *Bacillus cereus* in Milk and on Dairy Equipment. By KATHLEEN O. DONOVAN 131

The Investment Value of Continuous Culture. By R. ELSWORTH, R. C. TELLING and D. N. EAST. (With three figures) 138

The Value of the Association Escherichieae-Group D Streptococci in the Diagnosis of Contamination in Foods. By R. BUTTIAUX. (With one plate) . 153

Physical Aspects of Sterilizing Cotton Articles by Steam. By P. S. HENRY. (With four figures) 159

No. 2 (*issued* 31 *March*, 1960)

PAGE

Mycological Synthesis of Fat from Whey. II. Comparative Studies with Shaken and Stationary Cultures Using Selected Moulds. By P. WIX and M. WOODBINE. (With six figures) 175

The Suitability of Certain Obligately Anaerobic Nonsporeforming Enteric Bacteria, as Part of a More Extended Bacterial Association, as Indicators of 'Faecal Contamination' of Foods. By D. A. A. MOSSEL . . . 184

The Heat Resistance of *Escherichia coli* Cells from Cultures of Different Ages. By RUTH M. LEMCKE and HELEN R. WHITE. (With three figures) . . 193

The Biological Oxidation of Spent Gas Liquor. By EILEEN S. PANKHURST. (With five figures) 202

The Bacteriology of the Root Region of the Oat Plant Grown under Controlled Pot Culture Conditions. By H. A. LOUW and D. M. WEBLEY. (With two figures and one plate) 216

A Study of Soil Bacteria Dissolving Certain Mineral Phosphate Fertilizers and Related Compounds. By H. A. LOUW and D. M. WEBLEY . . . 227

Comparisons of Different Media for Counting Sugar Tolerant Yeasts in Concentrated Orange Juice. By M. INGRAM. (With three figures and one plate) 234

The Isolation of Coagulase Positive Staphylococci from Routine Composite Milk Samples. By A. H. WALTERS 248

The Changes with Age in the Resistance of *Escherichia coli* to Drying under Atmospheric Conditions. By RUTH M. LEMCKE 253

Studies on Aerosol Disinfection of Poultry Premises. By D. A. MCKENZIE, JEAN LAMBERT and J. GETTY 258

A Method for Counting the Total Bacteria in Ammoniated *Hevea* Latex Systems. By D. H. TAYSUM. (With one plate) 264

The Multiplication of Coli-Aerogenes Bacteria in Milk Stored at 3–5°. By J. J. PANES and S. B. THOMAS. (With one figure) 272

A Note on the Enumeration of Thermophilic Sulphate-Reducing Bacteria (*Clostridium nigrificans*). By A. W. BUFTON 278

The Influence of Rennet on Bacteriophage Infection in the Cheese Vat. By L. J. MEANWELL and NORAH THOMPSON 281

The Influence of Acidity on the Multiplication of Lactic Streptococcal Bacteriophage in Liquid Media. By NORAH THOMPSON. (With two figures) . . 287

The Differentiation of Bacterial Species by Paper Chromatography. VI. An Examination of the Heterofermentative Lactobacilli. By G. C. CHEESEMAN and M. T. SILVA. (With two figures and one plate). Appendix, A Note on the Source and Identification of the Strains of Heterofermentative Lactobacilli Submitted to Chromatographic Analysis. By M. ELISABETH SHARPE 294

The Differentiation of Bacterial Species by Paper Chromatography. VII. The Use of Electronic Computation for the Objective Assessment of Chromatographic Results. By G. C. CHEESEMAN and N. J. BERRIDGE. (With four figures) 307

PAGE

The Differentiation of Bacterial Species by Paper Chromatography. VIII. An Examination of *Lactobacillus lactis*, *L. leichmannii*, *L. delbrueckii* and *L. salivarius*. By M. T. SILVA and G. C. CHEESEMAN. (With one figure and two plates). Appendix, A Note on the Source and Identification of the Strains of Thermophilic Lactobacilli Submitted to Chromatographic Analysis. By M. ELISABETH SHARPE 317

No. 3 (*issued* 8 *July*, 1960)

An Approach to the Classification of the Lactobacilli. By M. ROGOSA and M. ELISABETH SHARPE 329

The Application of Chromatographic Techniques for the Identification of Species and Strains of Lactobacilli. By G. C. CHEESEMAN. (With three figures and two plates) 341

Lactobacilli of the Human Mouth. By G. H. G. DAVIS 350

Factors Affecting the Implantation of Lactobacilli in the Intestine. By H. B. HAWLEY, P. A. SHEPHERD and DOROTHY M. WHEATER 360

Micro-Organisms from Chicken Meat Related to both Lactobacilli and Aerobic Sporeformers. By MARGARET J. THORNLEY and M. ELISABETH SHARPE. (With one plate) 368

Some Special Characteristics of the Cider Lactobacilli. By J. G. CARR. (With two figures) 377

Contributions to Knowledge of the Malolactic Fermentation in Wines and Ciders. II. The Growth Promoting Effect of Yeast Extract on Lactic Acid Bacteria Causing Malolactic Fermentation in Wines. By H. LÜTHI and U. VETSCH. (With four figures) 384

Studies on Lactobacilli Causing Ropiness in Beer. By D. H. WILLIAMSON. (With five figures and one plate) 392

The Properties and Classification of Lactobacilli Isolated from Grass and Silage. By R. M. KEDDIE 403

Record of Proceedings at the meeting of the Society for Applied Bacteriology held in London on 14 *January*, 1959 i

ORIGINAL PAPERS

The Isolation of Virulent Staphylococci from Weekly Composite Milk Samples. By A. H. WALTERS i

The Multiplication of Coli-aerogenes Organisms in Milk held at Refrigerator Temperatures. By J. J. PANES and S. B. THOMAS i

A Test to Differentiate *Pseudomonas* from Other Gram-Negative Bacteria. By MARGARET J. THORNLEY i

Studies on Aerosol Disinfection of Poultry Premises. By D. A. MCKENZIE, JEAN LAMBERT and J. GETTY ii

PAGE

The Growth of *Penicillium chrysogenum* in Continuous Culture. By S. J. PIRT and D. S. CALLOW ii

A Two-Stage Continuous Culture Apparatus. By D. S. CALLOW and S. J. PIRT ii

Studies Arising from the Observation of Osmophilic Yeasts by Phase Contrast Microscopy. By M. PAMELA SCARR and ELISABETH COSTER . . . ii

SYMPOSIUM ON DIFFERENTIAL MEDIA

The Use of Antibiotics in Media for Assessing Bacterial Infection in Food Yeast. By M. FLEMING, N. H. BARNARD and L. A. ALLEN . . . iii

Selective Media Used in the Microbiological Examination of Sugar Products. By M. PAMELA SCARR iii

Selective Media for Yeasts and Bacteria. By F. W. BEECH and J. G. CARR . iii

The Enumeration and Identification of Some Types of Anaerobic Bacteria Occurring in Foods. By D. A. A. MOSSEL iii

Media for Lactobacilli. By M. ELISABETH SHARPE iii

Differential and Selective Media for the Faecal Streptococci. By ELLA M. BARNES iii

Differential Media for Sulphur Bacteria. By J. POSTGATE . . . iii

Culture Media for Fungi. By G. SMITH iii

Record of Proceedings at the meeting of the Society for Applied Bacteriology held in London on 7–9 July, 1959 iii

SYMPOSIUM ON THE LACTOBACILLI

An Approach to the Classification of the Lactobacilli. By M. ROGOSA and M. ELISABETH SHARPE iv

The Differentiation of Some Species and Strains of Lactobacilli by Chromatography. By G. C. CHEESEMAN iv

Lactobacilli of the Human Mouth. By G. H. G. DAVIS iv

Factors Affecting Lactobacilli and their Implantation in the Intestine. By H. B. HAWLEY, P. A. SHEPHERD and DOROTHY M. WHEATER . . iv

Some Micro-Organisms from Chicken Meat Showing Relationships to both Lactobacilli and to Aerobic Sporeformers. By MARGARET J. THORNLEY and M. ELISABETH SHARPE iv

The Anomalous Behaviour of Some Cider Lactobacilli. By J. G. CARR . iv

Some Studies on Lactobacilli Causing Malolactic Fermentation in Wines. By H. LÜTHI iv

Studies on Beer-Spoilage Lactobacilli. By D. H. WILLIAMSON . . . iv

The Properties and Classification of Some Lactobacilli Isolated from Grass and Silage. By R. M. KEDDIE iv

PAGE

ORIGINAL PAPERS

The Significance in Food Hygiene of *Lactobacillus bifidus*. By D. A. A. MOSSEL iv

Microbiological Aspects of the Genus *Microbacterium*. By PATRICIA A. TURBUTT, A. SEAMAN and M. WOODBINE v

The Early Development of Dairy Microbiology. By A. SEAMAN . . v

The Microbiological Assay of Thiamine — A Modified Method. By PAT M. SCHOLES v

A Note on the Maintenance of Assay Organisms as Freeze-Dried Cultures. By PAT M. SCHOLES and W. S. GREAVES v

Benzoate-Resistant Yeasts. By M. INGRAM vi

Bacteria of the *Aeromonas-Kluyvera* Group. By B. P. EDDY . . . vi

Enterococci in Calves' Faeces. By A. WILSSENS and A. DE VLEESCHAUWER . vii

The Behaviour of Individual Bacteria in the Lag Phase. By L. B. QUESNEL and G. C. WARE viii

Some Difficulties in the Radioactive Labelling of Streptomycin. By G. C. WARE viii

Record of Proceedings at the meeting of the Society for Applied Bacteriology held in London on 27 October, 1959 viii

Index of Authors ix

Index of Subjects xiii

No. 2, Vol. 21 of the Journal of Applied Bacteriology was issued on 13 April, 1959.

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THE EFFECT OF NISIN ON THE GROWTH OF CELLS AND SPORES OF *CLOSTRIDIUM WELCHII* IN GELATINE

By J. E. EASTOE and JOAN E. LONG

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SUMMARY: The action of various concentrations of nisin on the development of cells and spores from untreated and heated suspensions of *Clostridium welchii* added to gelatine has been investigated, using a tube colony count technique. The development of vegetative cells was prevented by the presence of 2 Reading Units (R.U.) of nisin/ml of the final culture medium, although the effective concentration may have been 6 R.U./ml. Approximately 40 R.U./ml prevented colony formation from spores. Gelatine containing nisin was dried. A considerable proportion of the activity of the antibiotic was still present after storage for 5 weeks.

In connection with the anaerobic spoilage of semi-preserved (pasteurized) foods such as canned hams the common occurrence of organisms of the genus *Clostridium* in edible gelatines manufactured in France and other European countries has been noted (Buttiaux & Flament, 1951; Buttiaux & Beerens, 1954; Buttiaux, 1955). These workers found that 58% of the samples examined were contaminated with 0·5–100 organisms/g. *Cl. welchii* was the most common anaerobe; it was present in 42% of the gelatines. To eliminate this cause of spoilage of food products a tentative upper limit of one clostridial organism/g was proposed.

Recently, Hawley (1957) suggested that it might be advantageous to add nisin to the grades of gelatine used in food manufacture, since it inhibits many spore forming anaerobes. The present investigation concerns the possibility of suppressing the development of clostridia in gelatine by the addition of nisin. Since no facilities were available for experiments on the control of spoilage of canned products, the survival of organisms added to gelatine containing nisin was studied under various conditions. *Cl. welchii* was used because Buttiaux found it the most common anaerobic contaminant of European edible gelatines; however, limited evidence suggests that this may not be true for British gelatines.

Materials and Methods

Organisms. *Cl. welchii*, Type A, NCIB 6125, was mainly used. In Robertson's cooked meat medium it produced approximately 10^8 cells/ml and normally some 10^6 spores/ml in 18–20 hr at 37°. This level of sporulation is high for this organism (Gibbs & Hirsch, 1956); occasionally the degree of sporulation was considerably lower. A few experiments were carried out with *Cl. bifermentans* NCIB 506. Stock cultures of both organisms were maintained in cooked meat at 2°.

Gelatine. One gelatine sample was used throughout. It was a long-limed, deionised hide gelatine having an isoionic point at pH 4·96, which was also its natural pH value. It contained approximately 5,000 aerobic bacteria/g and a few anaerobes, the number of which did not exceed 1/2 g, which is negligible in comparison with the numbers added for experimental purposes.

Nisin. A standard preparation of nisin, containing 10^6 Reading Units (R.U.)/g, kindly supplied by Mr. H. B. Hawley (Research Division, Aplin & Barrett, Ltd.), was used. An accurately weighed quantity was dissolved in sterile 0·01 N HCl and diluted as required with sterile distilled water. Two standard solutions were used, containing 120 and 5,000 R.U./ml respectively. The quantity of HCl in the nisin solution did not materially affect the pH of the gelatine solutions. The nisin concentrations quoted are those in the actual mixture in which the organisms grew. The concentrations in the 5% gelatine solution and air-dried gelatine were 3 and 60 times as large, respectively.

Preparation of inocula. Sterile cooked meat medium (in a screw-capped bottle) was inoculated with a loopful of organisms from a stock culture which had been stored at 2°, and incubated for 18–20 hr at 37°. After shaking and allowing to settle for 30 sec a portion of the supernatant suspension was withdrawn.

In experiments involving counts of vegetative cells, an initial dilution of approximately $1:5\times10^3$ was made by transferring a standard loopful of the supernatant to 9 ml of sterile quarter-strength Ringer's solution. From this three dilutions were made by means of sterile 1 ml pipettes, up to 1 in 5×10^6.

For experiments with spores, 1 ml of the supernatant was added to 9 ml of sterile quarter-strength Ringer's solution in a screw-capped bottle, which was then completely immersed in a water bath at 75° for 30 min to kill vegetative cells and activate the spores. Varying the heating time between 10 and 30 min did not markedly affect the counts, so the longer time was chosen to provide a safety margin. Heating in sealed glass tubes gave similar results but was less convenient.

Preparation and inoculation of gelatine solutions and addition of nisin. Portions of gelatine (10 g) were added to 200 ml of distilled water in 250 ml plugged conical flasks and, except when it was later necessary to set the gelatine solutions, sterilized by autoclaving at 15 lb/in.^{2} for 20 min. The solutions were cooled to 40° and mixed thoroughly. When desired, the pH was raised from 5·0 to 7·0: this required 2·5 ml of N NaOH.

The gelatine solutions were inoculated with 1 ml portions of the various dilutions of the cultures, and thoroughly mixed. Duplicate 10 ml portions from each flask were then poured into sterile plugged 6×1 in. test tubes for enumeration of the inoculum (see below). Appropriate small volumes (<5 ml) of nisin solution were then mixed with the gelatine solutions remaining in the flasks and duplicate 10 ml portions again poured for counts. More nisin solution was then added and further 10 ml quantities poured, and so on.

Method of counting organisms. To each 10 ml portion of 5% gelatine solution in its plugged 6×1 in. test tube, 20 ml of melted ferric sulphite-tryptone medium (Mossel *et al.* 1956) at 50° were added. After thorough mixing by rotating the tubes rapidly between the palms, the tubes were cooled in water to make the contents set,

and then about 10 ml of melted 1·5% agar were added. After incubation for 18 hr at 37° the dense black colonies were counted. If none was seen, the tubes were incubated for a further 24 hr and re-examined.

Nisin concentrations. The concentrations given below are those in the above tubes, i.e. in the mixture of 10 ml of gelatine solution (including the volumes of inoculum and of nisin solution added to the bulk of the gelatine) and 20 ml of ferric sulphite-tryptone medium.

Results

Preliminary experiments showed that *Cl. welchii* NCIB 6125 produced conspicuous dense black colonies in 18 hr at 37°. Far fewer colonies developed after the tubes had first been heated at 75° for 15 min, showing that vegetative cells survived after suspension in the gelatine solution.

It was also noted that fewer colonies developed from the 5% gelatine solutions than from similarly inoculated sterile water. This was subsequently traced to a pH effect; the gelatine, which was at pH 5·0, reduced the pH of the medium from 7·06 to 6·56, which is below the optimum for the organism. This effect could not be overcome by adding an excess of pH 6·90 phosphate buffer, as the volume required was too large. Instead the gelatine solution was brought to pH 7·0 with standard NaOH, and this resulted in a substantial increase in the number of colonies. Unheated solutions gave an approximately sixfold increase, while with heated solutions the numbers were three times as large.

Effects on vegetative cells of Cl. welchii *in gelatine solutions*

Two experiments were done, each at two inoculation levels, with the results shown in Table 1. The higher the nisin concentration the lower was the colony count, and the second experiment indicated that 2·0 R.U./ml of the final mixture would virtually eliminate an inoculum of *c.* 1000 cells/10 ml of 5% gelatine solution, while 1·0 R.U./ml

Table 1. *The effect of nisin in reducing the colony count of* Cl. welchii *in inoculated* 5% *gelatine* (10 *ml*) *mixed with Mossel's medium* (20 *ml*)

(inoculum not heat treated)

Expt no.	Numbers of colonies/tube* with nisin concentrations (R.U./ml in the final mixture) of:					
	0·00	0·04	0·20	0·50	1·0	2·0
1	*c.* 1000†	‡	‡	—	1, 2	—
	103, 128	79, 91	53, 46	—	0, 0	—
2	*c.* 1000†	—	—	54, 48	19, 20	3, 2
	104, 95	—	—	13, 8	9, 2	0, 0

* Duplicate tubes at each nisin level. † Based on next higher dilution.
‡ Too many colonies to count. —, Experiment not done.

would do the same with the smaller inoculum. However, in the first experiment the count was reduced to a similarly low level by 1·0 R.U./ml or perhaps less. It must be pointed out that because of the technique used in these experiments the cells

were exposed for short times (5 min at most) to nisin concentrations three times those recorded in Table 1, i.e. before the addition of the 20 ml of Mossel's medium. If nisin acts very quickly on vegetative cells, the concentration required for virtual elimination of that inoculum should be 6 R.U./ml.

Effects on Cl. welchii *spores in gelatine solution*

As shown in Table 2, between 8 and 40 R.U. of nisin/ml of the final mixture reduced an inoculum of *c.* 6,600 spores/10 ml of 5% gelatine solution to zero, and 8 R.U./ml were similarly effective on *c.* 550 spores/10 ml. The results of the two experiments were quite concordant, as shown by the colony counts with 2 R.U./ml and an inoculum of approximately 550–660 spores/10 ml. In experiment 4 duplicate tubes were stored at 2° for 24 hr before incubation, but this made little difference to the results. Apparently the nisin did not need to be given extra time to disperse in the final mixture. The same considerations about the effective nisin concentration apply here as with the vegetative cells.

Table 2. *The effect of nisin in reducing the colony count of* Cl. welchii *in inoculated* 5% *gelatine solution* (10 *ml*) *mixed with Mossel's medium* (20 *ml*)

(inoculum heated at 75° for 30 min before addition to gelatine solution)

Expt no.	Period of storage at 2° before incubation	Numbers of colonies/tube* with nisin concentrations (R.U./ml in the final mixture) of:			
		0	2	8	40
3	None	*c.* 6,600†	‡	18, 40	0, 0
		c. 660†	17, 16	—	—
4	None	*c.* 5,500†	‡	14, 6	—
		c. 550†	15, 14	1, 0	—
4	24 hr	*c.* 7,000†	127, 113	3, 3	—
		c. 700†	12, 19	0, 0	—

* Duplicate tubes at each nisin level. † Based on higher dilutions.
‡ Too many colonies to count. —, Experiment not done.

Effects on Cl. bifermentans *spores in gelatine solutions*

Difficulty was experienced in working with *Cl. bifermentans*, since a dense black fog was produced wherever more than a few colonies were present in a tube. The use of a shorter incubation period was tried, but the difficulty remained. However, no colonies grew when the nisin concentration was 8 R.U./ml of the final mixture, which suggests that *Cl. bifermentans* is as susceptible as *Cl. welchii*.

The stability of nisin during drying in gelatine solutions

The stability of nisin in gelatine solutions during evaporation in a current of air at or slightly above room temperature is of considerable practical interest. Since the stability of nisin in aqueous solution falls with increasing pH, it was considered advisable to investigate its stability in gelatine at two pH values, 5·0 and 7·0, the latter representing the upper limit of the range normally used in manufacture.

Concentrations which would give 8 and 40 R.U./ml of final mixture (i.e. 24 and 120 R.U./ml of gelatine) were established in unsterilized 5% gelatine solutions at both pH values. After the solutions had set, the gel was cut into slices which were dried in an air tunnel. These operations were carried out under clean but not aseptic conditions.

The dried gelatines were redissolved to give 5% solutions, inoculated with known numbers of *Cl. welchii* spores and 10 ml portions mixed with 20 ml of Mossel's medium to determine the survivors, and hence the approximate amount of nisin which had survived the drying process, by comparison with the effect of the same concentrations of nisin freshly added to gelatine solutions. The solutions from the gelatine dried at pH 5·0 were adjusted to pH 7·0 before the spores were added in order that conditions should be optimal for growth.

The results obtained are summarized in Table 3. No colonies developed where the original concentration of nisin in the gelatine solution was 120 R.U./ml, whether drying took place at pH 5·0 or pH 7·0. With 24 R.U./ml in the gelatine solution no organisms grew when the nisin had been freshly added but numbers representing 2–10% of the controls grew when the nisin had been dried with the gelatine. This indicated that some nisin had probably decomposed at both pH values, though this effect may not have been large since a few spores sometimes survived this concentration of freshly added nisin (Table 2).

Table 3. *The effect of drying in gelatine on the activity of nisin against* Cl. welchii *spores*

Numbers of colonies/tube* with original nisin concentrations in the gelatine solution of:						
0	24 R.U./ml			120 R.U./ml		
Undried	Undried	Dried, pH 5	Dried, pH 7	Undried	Dried, pH 5	Dried, pH 7
c. 1000†	0, 0	94, 125	46, 48	0, 0	0, 0	0, 0
94, 102	0, 0	4, 7	3, 1	0, 0	0, 0	0, 0

* Duplicate tubes at each level. † Based on next higher dilution.

The effect of nisin on Cl. welchii *spores during drying and storage of gelatine*

The numbers of clostridial spores originally present in gelatine liquors which survive the drying process and varying periods of subsequent storage are important, since they bear on the possibility of food spoilage occurring under special conditions. An experiment was therefore carried out to assess the effect of nisin on the numbers of added *Cl. welchii* spores which were viable before drying, after drying and after a period of 5 weeks storage of the dried gelatine. The concentrations used were equivalent to 1·6, 8·0 and 40 R.U./ml of the final mixture, i.e. *c.* 5, 24 and 120 R.U./ml of gelatine solution.

Unsterilized 5% gelatine solutions, previously adjusted to pH 7·0, were inoculated with 1 ml portions of unheated 10^{-1} and 10^{-2} dilutions of a *Cl. welchii* culture and

appropriate volumes of nisin were added where required. Portions (10 ml) of the solutions were poured into 6×1 in. tubes and heated at 75° for 15 min (to kill surviving vegetative cells) before colony counts were carried out. The remaining portions of the gelatine solutions were allowed to set, cut up and dried in an air tunnel. The dried gelatine was stored in sterile tins. When further counts of colonies derived from spores were to be made, the gelatine was first redissolved in sterile water to make 5% solutions. The inoculum was not heated in order that the added organisms might simulate a natural contamination, where vegetative cells might form spores during drying. The results are summarized in Table 4. There was no decrease in the number of spores, capable of development into colonies, in the absence of nisin, after drying or after storage for 5 weeks. At none of the nisin concentrations was a decreased count shown after drying compared with the initial count, while the number of spores surviving at concentrations of 1·6 and 8 R.U./ml increased, suggesting that the nisin was gradually decomposing and so becoming less effective in the final mixture. This effect might continue if the storage of the dried gelatine were prolonged. A level of 120 R.U./ml of gelatine solution (2,400 R.U./g of dry gelatine) reduced counts of the order of 1,000 to zero, except for one tube prepared from gelatine which had been stored for 5 weeks, in which one colony grew.

Table 4. *The effect of nisin on* Cl. welchii *spores during drying and subsequent storage of gelatine*

Numbers of colonies/tube* with different initial nisin concentrations (R.U./ml of final mixture)

Before drying				After drying				After 5 weeks' storage			
0	1·6	8	40	0	1·6	8	40	0	1·6	8	40
c. 1,200†	‡	40	0	‡	‡	109	0	‡	‡	>200	0
c. 1,200†	‡	16	0	‡	‡	78	0	‡	‡	>200	1
121	34	3	0	153	118	19	0	194	131	45	0
119	35	2	0	145	121	14	0	146	104	46	0

* Duplicate tubes at each nisin level. † Based on next higher dilution. ‡ Too many colonies to count.

Discussion

The precise effect of a given level of nisin may depend on variables such as the supply of nutrients and the pH. In this study it was attempted to make growth conditions as favourable as possible to the organism in order to provide the most stringent test conditions. The nutrients were abundant and the pH close to the optimum. The reduction of the pH of the medium by the gelatin resulted in diminished growth of both spores and cells of *Cl. welchii*, and this effect needs to be taken into account when the number of clostridial spores in gelatines is determined by methods such as that of Buttiaux & Flament (1951). Commercial edible gelatine is often supplied having a slightly acid reaction in order to minimize its susceptibility to microbial attack, and when tested for clostridia it should be brought to the pH of the medium by the addition of alkali. If all departures from optimum growth conditions result in a lowering of the concentration of nisin which inhibits clostridial

growth completely, then concentrations determined in a rich, highly anaerobic medium of optimum pH ought to be effective under all other conditions.

Gelatine is normally used in dilute solution, the concentrations ranging from say 1% to 5%. Since no enhanced effect of nisin occurs on drying with gelatine, while some decomposition probably takes place then and also during storage, it would appear desirable for the food manufacturer to add the nisin during the preparation of the gelatine solution. In a 2% solution a final level of 40 R.U./ml is required, which corresponds to 2000 R.U./g or 10^6 R.U./lb of dry gelatine. This would probably at present be uneconomic for general use, though where damage to an expensive product is possible it might be contemplated. Judicious addition of nisin by the user in special instances, rather than widespread employment by the gelatine manufacturer, would seem preferable.

This paper is published by permission of the Director and Council of the British Gelatine and Glue Research Association.

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THE INACTIVATION OF *BACILLUS SUBTILIS* SPORES IN PENICILLIN BY GAMMA RADIATION

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SUMMARY: Sodium benzylpenicillin, contaminated with *Bacillus subtilis* spores by freeze-drying a suspension of spores in an aqueous penicillin solution (*c.* 50% w/v), was exposed to gamma radiation and a 70-tube dilution method was used to determine the surviving spores after various doses. The correlation coefficient between $\log_{10}$ percentage survival and dose was −0·9523. The regression of the former on the latter was calculated and the decimal reduction dose found to be $20{\cdot}2 \times 10^4$ rads. The regression and the decimal reduction dose were similar to those obtained when suspensions of spores in distilled water were irradiated.

Existing methods of obtaining sterile pharmaceutical products involve the use of heat, filtration and aseptic manipulation, which contribute considerably to the cost of the product. An economic alternative to present methods would be a welcome asset to the pharmaceutical industry (Jones, 1953). Many workers have produced radiation inactivation curves for a variety of micro-organisms and a few attempts have been made to sterilize antibiotics by gamma radiation (Nickerson, Proctor & Goldblith, 1953; Controulis, Lawrence & Brownell, 1954) and high-energy electrons (Miller, 1954). Detailed quantitative results for antibiotic sterilization have not so far been published.

The experiments described below were designed to follow the inactivation of *Bacillus subtilis* spores when evenly distributed in sterile sodium benzylpenicillin. One experiment was also carried out to follow the inactivation of spores of this organism suspended in distilled water.

Methods

Irradiation. This was provided from a 200 curie cobalt-60 source of similar design to one described by Gibson & Pearce (1957) which operates at the Atomic Energy Research Establishment, Harwell. The cobalt is in the form of five 12 mm pellets of about 40 curies activity each, canned in aluminium to prevent corrosion and encased in a stainless steel tube about 6 cm long. In the investigation described, the irradiation cells were arranged around the source in a revolving turntable to ensure even and reproducible dose rates. The contaminated penicillin was irradiated in 5 ml glass tubes, sealed in air, each containing *c.* 0·5 g of penicillin. Aqueous spore suspensions were irradiated in similar sealed tubes each containing 1 ml of spore suspension.

A chemical method (Miller & Wilkinson, 1952) was used for dosimetry. This is based on the oxidation of ferrous to ferric ions, the solution being 0·8 N in sulphuric acid and 10^{-3} M in ferrous ion.

Contaminated penicillin. To prepare this, the 72 hr growth on a 30° nutrient agar slope of *Bacillus subtilis* (NCTC 2116) was washed off with distilled water, the suspension heated to 85° for 10 min, and washed six times in distilled water by centrifugation. This was then added to a solution of 20 g of sodium benzylpenicillin (Glaxo Laboratories, Ltd.) in 40 ml of distilled water and, after mixing, freeze dried in a 500 ml flask for 7 hr. The contaminated penicillin was stored at −18° in a desiccator and mechanically mixed before use. The moisture content under these conditions, as determined by drying to constant weight at 105° in a vacuum oven, was 3·5%.

The spore suspensions used in expt. 4 were similarly grown, heated and washed, and the spore numbers estimated microscopically. The suspension was diluted to *c.* 1000 spores/ml and used the same day.

Viable spore counts

Preparation of dilutions. For experimental purposes the contaminated penicillin was distributed in sealed 5 ml glass tubes each containing *c.* 0·5 g of penicillin. To assay the number of viable spores/g the contents of a tube were transferred to a weighed sterile McCartney bottle; after re-weighing, sterile distilled water was added at the rate of 9·5 ml/g of penicillin to give a solution of 0·1 g/ml. This was diluted 1 to 50, then 1 to 49, and 1 ml of penicillinase (Wellcome) added to 49 ml of the latter dilution. Thus excess penicillinase was present. Also, the phenol present as a preservative in the penicillinase was adequately diluted. This dilution was left at room temperature for 2 hr, after which Heatley cup tests failed to show any residual antibiotic activity. One further suitable dilution was made and used as the starting point for the 70-tube test.

Method of counting. In early experiments, in which conventional nutrient plate colony counts after 3 days at 30° were used, the viable count apparently rose as the dilution increased in both control and irradiated samples. A tenfold dilution of inocula increased the viable count by from 30 to 110%. This was not due to the breaking up of clumps, as mechanical agitation of more concentrated suspensions failed to increase the colony counts, and was possibly due to the presence of an inhibitor of spore germination. It was therefore decided to use a tube dilution method, so that any such inhibitors would be diluted. The method was based on the tables of W. L. Stevens (see Fisher & Yates, 1953), and it was not affected by dilution, as shown below.

It utilized a block of 70 tubes of 5 ml of Oxoid CM 1 nutrient broth arranged in seven rows of ten, each row receiving inocula (1 ml) twice as dilute as the previous row. The dilution made after the addition of penicillinase and inactivation of the penicillin, was used as the inoculum for the first row of tubes. The degree of dilution was so arranged that about half the tubes would eventually show growth. Subsequent rows received doubling dilutions as inocula.

After inoculation the tubes were incubated at 30°. Each day the total number positive was recorded, and the tubes replaced on a different shelf of the incubator to minimize any positional effect. Most of the positives were apparent on the 4th day. Further positives were never noted after the 9th day and as a routine, therefore, the total number of positives on the 10th day was recorded.

The number (λ) of organisms/tube of the highest concentration was then calculated according to the formula $\log_{10}\lambda = x\log_{10}a - K$, where x is the mean fertile level (total number of positive tubes divided by the number of tubes/row, in this case 10), a is the dilution factor, in this case 2, and K is found by reference to Stevens' tables. Since the variance of $\log_{10}\lambda$ is given by $\log_{10}2$ times $\log_{10}a$ divided by the number of tubes/row, in the present work the variance of $\log_{10}\lambda$ is $(\log_{10}2)^2/10$ and the mean standard error is $\log_{10}2/\sqrt{10}$, or 0·095. Thus in an assay in which half the tubes were positive, λ would be 4·73/ml with a range of 3·05–7·32 (P=0·05).

Tests of the method. Two samples of contaminated penicillin were examined. After dilution, addition of penicillinase and standing for 2 hr, each was diluted a further 100 times and a 70-tube assay conducted. The numbers of fertile tubes were 35 and 36, giving values of $\log_{10}\lambda$ of 0·6745 and 0·7066, which are well within one mean standard error (0·095) of each other. Two samples of contaminated penicillin which had been irradiated for 2 hr (156,000 rads) were then tested. After standing with penicillinase, each was diluted 25 times. The values of $\log_{10}\lambda$ were 0·9002 and 0·9323, which again show agreement well within one mean standard error.

That increasing dilution had no effect on the recovery of spores from contaminated penicillin when the 70-tube method was used, was shown in the following experiment. A sample was diluted and penicillinase added as usual. After standing, dilutions of 1 in 20 and 1 in 100 were made, each being used as the starting point for a 70-tube assay. The fertile tubes numbered 56 and 36 respectively, giving values for $\log_{10}\lambda$ of 1·3936 and 0·7066. After correcting the latter for the dilution factor it became 1·4056, in excellent agreement with the value obtained using the more concentrated solution.

The viable counts of the spore suspensions in distilled water were carried out by the same method, using a suitable dilution as the starting point for the 70-tube test.

Results

Three independent experiments were conducted to determine the death rate of *Bacillus subtilis* spores in penicillin when irradiated at 1,300 rads/min. Sealed glass tubes containing *c.* 0·5 g of contaminated penicillin were irradiated, single tubes being removed at intervals up to 240 min. One tube received no radiation and served as a control. The viable spores were then estimated and the survivors expressed as percentages of the number in the control tube. The results are shown in Table 1 and Fig. 1a. As the conditions were similar in each experiment the results were combined and a product moment correlation coefficient r calculated, relating dose to $\log_{10}$(% survival). Its value was −0·9523, which signifies a very high negative correlation between dose and $\log_{10}$(% survival). Using the 't' test, the probability of this correlation coefficient occurring by chance proved to be <0·001. The linear regression

of $\log_{10}$(% survival) on dose was calculated and is shown in Fig. 1a. The standard error for an estimate of $\log_{10}$(% survival), given the dose, is 0·1521 and the standard error of the dose, given the $\log_{10}$(% survival), is 29,300 rads.

Table 1. *The effect of gamma radiation on the number of viable* B. subtilis *spores in contaminated penicillin*

Expt no.	Dose (10^4 rads)	Spore survival	
		10^3/g	%
1	0·00	26,930	100·0
	3·89	23,780	88·3
	7·79	13,150	48·8
	11·68	11,490	42·7
2	0·00	21,390	100·0
	7·79	9,205	43·0
	15·57	2,554	11·9
	23·36	890	4·2
3	0·00	11,820	100·0
	7·79	5,499	46·5
	15·57	4,967	42·0
	23·36	1,346	11·4
	31·14	368	3·1

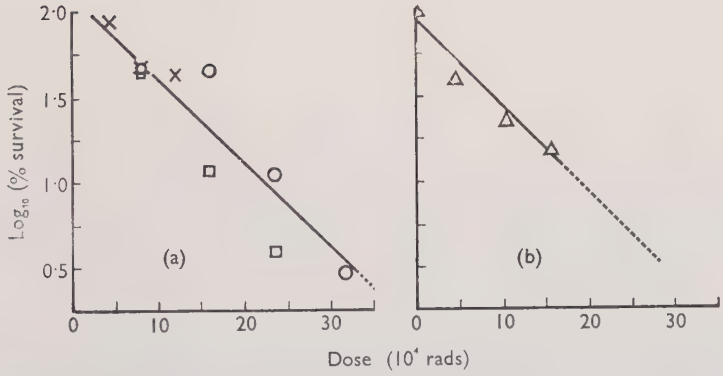

Fig. 1. The relationship between the dose of gamma radiation and the surviva of *B. subtilis* spores in contaminated penicillin (a) and in aqueous suspension (b).
Crosses, expt. 1; squares, expt. 2; circles, expt. 3; triangles, expt. 4. The formulae of the regressions of y ($=\log_{10}$ % survival) on x ($=$dose in rads) are: for spores in penicillin, $y = 2{\cdot}058 - 0{\cdot}000004944\,x$; for spores in aqueous suspension, $y = 1{\cdot}927 - 0{\cdot}000005152\,x$.

A further experiment was conducted to determine the death rate of an aqueous suspension of *B. subtilis* spores prepared as for the penicillin contamination and diluted in distilled water without freeze-drying. The results are shown in Table 2 and Fig. 1b. The correlation coefficient between $\log_{10}$(% survival) and dose was −0·9774, with a probability due to chance of 0·03. The regression was also calculated (see Fig. 1b). The probability that the slope of this regression and that of the

regression for spores in penicillin are the same is 0·8, and the use of Fisher's Z transformation showed the difference between the two correlation coefficients to be less than one standard error.

Table 2. *The effect of gamma radiation on the viability of* B. subtilis *spores in aqueous suspension* (*Expt* 4)

Dose (10^4 rads)	Spore survival	
	No./ml	%
0·00	547	100·0
4·80	214	39·1
10·38	123	22·5
15·57	82	15·0

Discussion

The analysis of the data from the experiments described has shown that the death rate of *B. subtilis* spores distributed in penicillin is exponential with increasing gamma irradiation, at least up to doses of *c.* 30×10^4 rads. Although these data apply strictly only to the dose rate of 1,300 rads/min, it is generally accepted, and has been borne out in our experience, that inactivation is independent of rate. However, Sutton & Rotblat (1957) showed that certain chemical reactions become affected by dose rate when this approaches 10^7 rads/sec, and suggested that this phenomenon is due to overlapping of 'reaction zones'. It would therefore be unwise to assume that the present inactivation data would apply at very high dose rates.

It is anticipated that the most useful value to be derived from the data is the 'decimal reduction dose', i.e. the dose to give a mortality of 90%. For spores distributed in penicillin this was $21·4\times10^4$ rads. Thus if the degree of contamination was not expected to exceed 10^6 spores/g and a survival level of 1 spore/100 g was acceptable, the equivalent of eight decimal reduction doses could be given, assuming that the linear regression can be extrapolated to higher doses. The LD_{50} was $7·3\times10^4$ rads.

There is considerable evidence that the recovery of viable organisms after irradiation varies with differing media and incubation temperatures and it is not suggested that the conditions used (nutrient broth at 30°) necessarily gave the highest possible yield. It is also probable that some cells which fail to yield visible growth still possess many of the activities of living organisms. However, from the practical aspect of antibiotic sterilization, unless gross contamination is present it suffices that the cells are no longer capable of reproduction.

The agreement between the results for the freeze dried spores in penicillin and the spores suspended in distilled water supports the evidence of Bellamy & Lawton (1955) that the radiation resistance of *B. subtilis* spores is not appreciably altered by freezing or drying. However, Tallentire (1958) found that thoroughly freeze dried spores of *B. subtilis* were considerably more sensitive than spores which had been spray dried. Drying of other organisms (Stapleton & Hollaender, 1952; Moos, 1952) appears to increase radiation resistance. The present data were derived from three experiments with freeze dried spores and one with an aqueous spore suspension and

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MYCOLOGICAL SYNTHESIS OF FAT FROM WHEY. I. PRELIMINARY STUDIES WITH STATIONARY CULTURES

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SUMMARY: Forty mould species, 30 of which were from the genera *Aspergillus*, *Penicillium*, *Fusarium* and *Mucor*, were examined for their ability to utilize the lactose in whey and to produce fat in the mycelium in stationary cultures.

In terms of lactose consumed and weight of mycelial felt produced the most promising species were *A. ustus*, *P. frequentans*, *P. oxalicum* and *P. notatum*. The first three of these warrant further study as fat producers.

The processing of plant and animal products results in the production of large volumes of sugar-rich fluids, such as whey from the cheese industry, which must be utilized or disposed of. Wix & Woodbine (1958) reviewed the literature on the disposal and utilization of whey, and it seems that past methods of disposal have often caused river pollution, while the market for whey as a raw material is limited.

The use of micro-organisms for the conversion of waste sugar residues into protein or fat on an industrial scale has been closely associated with the 1914–18 and 1939–45 wars, when necessity rather than economy often determined the employment of industrial processes. The possible conversion of whey to substances more acceptable to commerce has received much attention, and Woodbine (1951, 1959) reviewed the production of fat by yeasts, moulds, algae and bacteria and by chemical synthesis; Steiner (1957) also reviewed the subject of fats.

The object of the present investigation was to find moulds which would not only reduce the lactose content of whey, thus lowering its biological oxygen demand and so facilitating its disposal, but also during their growth form useful quantities of fat in their mycelium. The utilization of lactose and the production of fat are complementary to one another.

Experimental Methods

Preparation of the medium. Whey was autoclaved at 8 lb pressure for 15 min in 540 ml screw capped blood transfusion bottles. The precipitated proteins were allowed to settle, after which 50 ml quantities of the supernatant were placed in 250 ml Erlenmeyer flasks and autoclaved a second time.

Analysis of whey. The percentages of water, total solids, fat, and ash were determined by standard methods. Lactose was determined by the ferricyanide reduction method of Hagedorn & Jensen (1923) as modified by Blackwood (1934).

A micro-Kjeldahl method was used in conjunction with the Markham apparatus to determine nitrogen (Markham, 1942; Yuen & Pollard, 1953).

Inoculation and incubation. Each 50 ml quantity received 0·5 ml of spore suspension. Incubation was at 28·5±0·5° in a controlled temperature room.

Harvesting. The flasks were autoclaved at 8 lb pressure for 15 min and the mould felts filtered off on a Buchner filter, washed, and dried to constant weight at 70°.

Analysis of culture fluids and dried mould felts. The pH values of the uninoculated media and culture fluids were determined using pH papers (Johnsons Ltd., London, N W 4) or by a Mullard pH meter. Lactose and nitrogen were determined as before, the dried felts being powdered in a coffee mill before analysis. Fat was estimated by a Soxhlet method using the 24 unit semi-micro fat extractor of Wix & Hopton (1957).

Mould species. The species of moulds used are listed in Table 1. They were largely those used by Woodbine (1951) and Woodbine, Gregory & Walker (1951), chosen originally after an exhaustive search of the literature for species known to have fat producing potentialities. Of the 40 species used, 10 were *Aspergillus*, 10 *Penicillium*, 5 *Mucor* and 5 *Fusarium*, while the rest were from the genera *Mortierella*, *Oospora* (now *Geotrichum*) (2), *Paecilomyces*, *Rhizopus* (2), *Ustilago*, *Zygorrhynchus*, *Eremothecium* and *Cladosporium*. The strains were kept on beer wort agar slopes and subcultured at intervals of 2–3 months.

Table 1. *A list of the moulds studied**

Aspergillus spp.		*Fusarium* spp.	
A. clavatus Desm.	T. K. Walker	*F. lini* Bolley	T. K. Walker
A. flavipes (Bain. & Sart.) Thom. & Church	52107	*F. bulbigenum* Cooke & Massee var. *lycopersici* (Brushi) Wollenw.	45647
A. flavus Link ex Fr.	45542	*F. graminearum* Schw.	49105
A. ustus (Bain.) Thom & Church	40414	*F. oxysporum* Schlecht. (ex. Fr.) sensu Snyder & Hansen	52989
A. nidulans Eidam	T. K. Walker	*F. lateritium* (Nees ex. Fr.) sensu Snyder & Hansen	49897
A. niger v. Tiegh.	17454	*Mucor* spp.	
A. fischeri Wehm.	54402	*M. circinelloides* v. Tiegh.	31800
A. candidus Link ex. Fr.	15963	*M. ramannianus* Moller	35044a
A. oryzae (Ahlb.) Cohn	44242	*M. plumbeus* Bon.	38798
A. terreus Thom	45543	*M. mucedo*	29663
		M. albo-ater Naumoff	B.C.C.
Penicillium spp.		*Species of other genera*	
P. javanicum van Beyma	T. K. Walker	*Mortierella hygrophila* Linnem	45945
P. notatum Westl.	15378	*Oospora lactis* (Fresen.) Sacc.	50119
P. frequentans Westl.	40234	*Paecilomyces varioti* Bain.	17457
P. spinulosum	T. K. Walker	*Rhizopus nigricans* Ehrenb.	42844
P. funiculosum Thom	52740	*Rhizopus oryzae* Went & Geerl.	50109b
P. oxalicum Currie & Thom	39750	*Ustilago maydis* (DC) Corda	35676
P. piscarium Westl.	57233	*Zygorrhynchus moelleri* Vuill.	29216
P. purpurogenum var. *rubri-sclerotium* Thom	40035	*Eremothecium ashbyi* Guill.	14783
P. roqueforti Thom	53562	*Geotrichum candidum* Link	46237
P. soppii Zal.	T. K. Walker	*Cladosporium herbarum* (Pers.) Link ex Fr.	55216

* With the exception of the 6 cultures received from Professor T. K. Walker and one, marked B.C.C., from the Baarn Culture Collection, all the strains came from the Commonwealth Mycological Institute and the numbers given are those of that culture collection.

Results

Composition of whey. Ten samples were examined and the mean values (% w/v) were: water, 93·43; lactose, 5·16; fat, 0·10; protein, 0·38; lactic acid, 0·34; ash, 0·54. The percentage composition of the ash was given by Cook (1958) as potassium, 0·18; sodium, 0·05; magnesium, 0·02; calcium, 0·05; phosphorus, 0·04 and chlorine, 0·11.

Table 2. *Lactose utilization and weight of mycelium produced by 40 mould species after 42 days growth in whey*

	Lactose used		Felt weight	Economic coefficient*
	(g/l.)	(%)	(g/l.)	
Aspergillus clavatus	31·8	51·3	13·4	42·1
A. flavipes	50·2	80·9	18·8	37·5
A. flavus	40·8	65·8	16·4	40·2
A. ustus	61·4	99·5	21·0	34·0
A. nidulans	30·0	48·4	11·2	37·3
A. niger	18·8	30·3	17·0	90·4
A. fischeri	29·6	47·7	17·6	59·5
A. candidus	10·8	14·2	14·6	135·2
A. oryzae	60·8	98·2	16·8	27·6
A. terreus	36·4	58·7	19·2	52·7
Fusarium lini	24·4	39·4	13·4	54·9
F. bulbigenum var. *lycopersici*	29·6	47·7	14·6	49·3
F. graminearum	22·2	35·8	17·2	77·5
F. oxysporum	33·8	54·5	16·4	48·5
F. lateritium	31·8	51·3	18·0	56·6
Penicillium javanicum	42·8	68·9	17·8	41·6
P. notatum	60·8	98·2	13·6	22·4
P. frequentans	60·6	97·6	19·0	31·4
P. spinulosum	39·8	64·1	21·0	52·8
P. funiculosum	47·6	76·6	17·8	37·4
P. oxalicum	57·0	91·9	23·2	40·7
P. piscarium	15·2	24·5	11·2	73·7
P. purpurogenum var. *rubri-sclerotium*	60·6	97·6	22·2	36·6
P. roqueforti	30·8	49·7	12·8	41·6
P. soppii	8·2	13·2	10·2	124·4
Mucor circinelloides	8·8	14·2	6·2	70·6
M. ramannianus	59·8	96·4	14·0	23·4
M. plumbeus	4·4	7·1	9·0	204·5
M. mucedo	4·0	6·5	6·0	150·0
M. albo-ater	2·6	4·2	5·8	223·1
Mortierella hygrophila	8·6	13·9	5·8	67·4
Oospora lactis	5·8	9·3	8·0	137·9
Paecilomyces varioti	60·2	97·0	15·2	25·2
Rhizopus nigricans	8·6	13·9	5·2	60·5
Rhizopus oryzae	4·4	7·1	7·0	159·1
Ustilago maydis	26·0	41·9	14·6	56·2
Zygorrhynchus moelleri	7·8	9·3	9·2	117·9
Eremothecium ashbyi	7·0	10·9	8·6	122·9
Geotrichum candidum	8·4	13·5	9·0	107·1
Cladosporium herbarum	11·2	18·1	10·6	94·6

* The weight of felt produced in g/100 g of lactose utilized.

Preliminary survey of moulds

The results of tests on all 40 moulds are given in Table 2. Only 22 of the moulds used over 40% of the lactose in 42 days. Of these, 8 species (*P. oxalicum*, *A. ustus*, *P. notatum*, *M. ramannianus*, *P. frequentans*, *P. purpurogenum* var. *rubri-sclerotium*, *Paecilomyces varioti* and *A. oryzae*) used over 90% and were selected for further study.

Experiments with selected moulds

The eight selected moulds were grown in quintuplicate for 10 days in whey, uninoculated control flasks of which contained, at harvesting time, lactose, 6·0% and nitrogen, 0·06% and had a pH value of 6·6. The results are given in Table 3. Only three of the eight species used over 50% of the lactose in the 10 day growth period and one other used just over 25%.

Table 3. *Lactose utilization and weight of mycelium produced by 8 selected mould species after 10 days growth in whey**

	Final pH	Lactose used (g/l.)	Lactose used (%)	Felt weight (g/l.)	Economic coefficient†
P. oxalicum	6·7	41·8	71·8	18·4	44·0
A. ustus	8·2	33·2	55·0	12·8	38·6
P. notatum	7·0	32·6	54·0	11·3	34·7
M. ramannianus	5·2	16·0	27·5	6·0	37·5
P. frequentans	7·2	6·0	9·9	6·4	106·7
P. purpurogenum var. *rubri-sclerotium*	7·5	3·6	6·2	7·6	211·1
Paecilomyces varioti	7·9	2·6	4·5	2·8	107·7
A. oryzae	9·1	1·2	2·0	4·2	350·0

* Initial pH, 6·6; lactose, 6·0%; nitrogen, 0·06%.
† Weight of felt as g/100 g of lactose utilized.

Periodic harvesting experiments

In further experiments the best 21 species in order of ability to utilize lactose (Table 2) were used in periodic harvesting experiments. Each species was grown in six sets of three replicate flasks of whey which initially contained 5% of lactose and 0·06% of nitrogen and had a pH value of 6·5. For each species there were also six uninoculated controls. At each harvesting three replicate flasks and one control flask were taken. The 8 best species were harvested after 5, 10, 15, 21, 25 and 30 days while the remaining 13, which utilized lactose more slowly, were harvested after 5, 12, 18, 25, 32 and 39 days. The results are based on a comparison with the control flasks at each harvesting, which minimizes errors due to evaporation losses during incubation. In lengthy periods at 28·5±0·5° these losses may amount to as much as 10%. The results of these experiments are summarized in Tables 4 and 5. From Table 4 it can be seen that 11 strains reached the stage of maximum fat production before reaching maximum felt weight, four reached these stages at the same time, and five others did not reach the stage of maximum fat production until after the maximum felt had been produced. One species gave an indeterminate result. In terms

of felt weight, *P. purpurogenum* var. *rubri-sclerotium* (17·6 g/l.), *P. frequentans* (17·4 g/l.), *P. oxalicum* (17·0 g/l.), *P. javanicum* (16·4 g/l.), and *A. ustus* (15·6 g/l.) were the most promising. Table 5 shows that the best four on the basis of lactose consumed were *A. ustus* and *P. oxalicum*, both of which used all the lactose in 15 days, *P. notatum*, which used 97·3% in 30 days and *P. frequentans*, which used 93·2% in the same period.

Rippel (1940) gave 15 as the maximum figure for the fat coefficient, defined as the weight of fat produced in g/100 g of sugar consumed. In this work only *F. lateritium* approached that figure and only eight other species had values greater than 1.

Table 4. *The production of mycelial felt and fat by 21 species of moulds grown in whey*

Mould species	Yield (g/l.) of		Time (days) for maximum production of		Maximum fat content (%)
	Felt	Fat	Felt	Fat	
A. ustus	15·6	1·37	35	15	9·95
P. frequentans	17·4	0·20	25	21	1·19
Mucor ramannianus	7·8	0·14	>30	21	2·78
A. flavipes	10·2	0·29	>39	25	3·69
P. spinulosum	3·8	0·04	>39	18	1·68
A. terreus	11·4	0·26	>39	18	2·55
F. oxysporum	12·4	0·55	>39	32	5·47
A. clavatus	6·4	0·13	>39	32	2·13
P. roqueforti	6·8	0·04	32	18	0·70
A. fischeri	7·2	0·07	>39	18	1·53
F. bulbigenum var. *lycopersici*	5·8	0·08	32	18	1·69
P. notatum	9·8	0·51	15	15	5·20
A. nidulans	12·2	0·61	32	32	5·01
Paecilomyces varioti	5·0	0·03	15	15	0·57
P. funiculosum	10·0	0·14	32	32	1·44
P. purpurogenum var. *rubri-sclerotium*	17·6	0·61	25	>30	3·89
P. oxalicum	17·0	0·29	15	>30	2·40
P. javanicum	16·4	0·25	32	>39	1·62
F. lateritium	9·0	1·74	25	39	27·17
A. flavus	6·6	0·15	25	>39	2·23
A. oryzae	10·2	0·48	>30	>30	4·75

With increasing efficiency of lactose utilization there was a fall in the economic coefficient (the weight of mycelial felt produced expressed as g/100 g of lactose utilized). The 10 species which utilized over 50% of the lactose had economic coefficients between 10·8 and 29·6. This range is similar to that obtained by Woodbine (1951), Garrido, Woodbine & Walker (1953) and Abson, Khan & Walker (pers. comm. 1956).

The general tendency during these experiments was for the pH to rise. The metabolism of lactose, at least by some moulds, would be expected to produce organic acids: this was observed in later experiments, but the buffering action of the whey would reduce their effect on the pH. Some cultures consumed lactose with no increase

in felt weight and no alteration in the pH of the medium. These species may have been effecting complete oxidation.

More detailed experiments were done with the four best lactose utilizers, *A. ustus*, *P. notatum*, *P. frequentans* and *P. oxalicum*. The whey contained 5% of lactose and 0·06% of nitrogen. Two initial pH values, 6·4 and 5·8, were used in two series of experiments. In both series *A. ustus* and *P. notatum* gave steady increases of felt and fat until, towards the end of the growth period, there were signs of autolysis and a corresponding fall in the felt and fat weights. Autolysis occurred in some cases before all the lactose had been consumed. *A. ustus* showed a good fat producing potential while *P. notatum* showed little ability. *P. frequentans* and *P. oxalicum* gave felt weights comparable with those of *A. ustus*, and although there was little fat produced in the early stages of growth, there was a sharp rise in the fat content at the end of the growth period.

Table 5. *Lactose utilization by* 21 *mould species grown in whey, and their economic and fat coefficients*

Mould species	Lactose used (g/l.)	Lactose used (%)	Economic coefficient	Fat coefficient§ at maximum fat %
Faster species*				
A. ustus	65·2	100‡	22·8	**3·2**
P. oxalicum	65·2	100‡	18·9	0·5
P. notatum	63·4	97·3	11·2	1·1
P. frequentans	60·8	93·2	26·9	**0·4**
P. purpurogenum var. *rubri-sclerotium*	58·6	89·9	26·9	1·1
M. ramannianus	37·6	57·7	20·8	0·7
A. oryzae	31·4	48·2	32·4	1·5
Paecilomyces varioti	9·0	13·8	45·1	0·2
Slower species†				
P. javanicum	52·0	86·4	29·6	0·5
A. flavus	61·6	84·6	10·8	0·2
A. flavipes	58·6	80·5	17·3	1·2
F. bulbigenum var. *lycopersici*	41·6	57·2	13·3	0·8
A. terreus	35·2	48·4	32·2	1·0
A. clavatus	34·2	46·9	19·0	0·4
A. nidulans	28·2	38·7	37·6	2·1
P. funiculosum	21·2	35·2	43·0	0·7
F. oxysporum	20·8	34·5	59·3	3·2
A. fischeri	18·2	25·0	39·3	1·2
F. lateritium	12·8	21·3	49·9	13·6
P. roqueforti	11·8	19·6	45·8	0·3
P. spinulosum	18·0	3·0	211·0	1·0

(Data obtained from periodic harvesting experiments.)

* Faster species, data obtained after 30 days. † Slower species, data obtained after 39 days.

‡ This value occurred at 15 days. § Expressed as g fat/100 g lactose utilized.

A. ustus and *P. notatum* reached a stage of maximum felt weight and fat percentage at the same time. *P. frequentans* and *P. oxalicum* had an initial stage of growth with little fat formation, followed by a stage of fat accumulation after the maximum felt weight had been reached. This is consistent with the work of Hopton & Woodbine

(1958), who noted that some yeasts are inherently good fat producers while others form predominantly protein and carbohydrate. The final results of these experiments are given in Table 6. Although in series 2 the growth periods were shorter, the amounts of felt produced were very little different from those in series 1 and less lactose was used to produce virtually the same weight of felt. Because of their period of delay in fat synthesis, *P. frequentans* and *P. oxalicum* formed fat during the period of active synthesis at a much faster rate than the other two moulds.

Table 6. *The results of further experiments with the four best lactose utilizers*

Mould species	Incubation period (days)	Lactose used (%)	Felt weight (g/l.)	Final fat (%)	Economic coefficient
Series 1					
A. ustus	38	99·5	13·0	15·5	15·3
P. notatum	38	62·2	6·4	1·3	12·0
P. frequentans	38	83·4	13·8	4·9	19·3
P. oxalicum	38	85·3	12·2	4·8	16·7
Series 2					
A. ustus	31	69·7	12·2	14·7	21·9
P. notatum	25	34·4	5·0	0·7	21·0
P. frequentans	31	63·2	12·2	7·7	24·2
P. oxalicum	25	59·5	13·4	9·9	32·5

Discussion

These experiments have shown that with many of the 40 moulds examined, periods of 30–40 days growth in stationary culture are needed to attain a useful reduction in the lactose content of whey, though sometimes complete utilization was effected in 15 days. It is assumed that species giving high economic coefficients in the earlier stages of growth were using nutrients other than lactose. In the periodic harvesting experiments the high economic coefficients of the initial growth phase fell to normal levels as growth proceeded. This aspect of growth in a natural medium, the absolute composition of which is difficult to determine, is inevitable. There is, for example, the possibility of lactic acid being used as a carbon source. In earlier work it had been found difficult to prepare media to simulate whey; lactose, for example, was not readily accepted as a carbohydrate source and it is not possible in a synthetic medium to incorporate the growth factors present in whey and there seems little doubt that, even after heat treatment, such factors must have some effects (see Cooperman *et al.* 1946; Peeler & Norris, 1951; Huff *et al.* 1950).

As regards fat production, the results support the view of the majority of previous investigators that fat is probably a reserve food material (see Perrier, 1905; Belin, 1926; Fink, Haehn & Hoerburger, 1937; Price *et al.* 1943; Foster, 1949), on the ground that when the carbohydrate supply becomes limiting the fat is used up. However, Kordes (1923) and Geffers (1937) considered fat to be an excretory product. The criterion of carbohydrate utilization is, however, satisfactory for the selection of fat-producing moulds, since the amount of fat produced is a function of the carbohydrate present (Belin, 1926; Terroine & Bonnet, 1927; Bohn, 1931).

Walker (pers. comm. 1956) stated that fat production does not occur in cultures where the pH falls, because sugars are metabolized to organic acids. In these experiments the pH tended to rise. This is interesting, as Thimann (1955) found that *Oospora lactis* caused acid whey to become neutral and then alkaline, due to its activities in utilizing proteins and deaminating amino acids, although the whey buffering effects will tend to oppose pH changes. From the work of other investigators it would seem that the pH ranges obtaining in the present work were favourable to fat production. Lockwood *et al.* (1934) stated that the range 3·1–6·8 was favourable and Kleinzeller (1948) gave it as 3·5–6·0. However, neutral or slightly alkaline conditions were considered most favourable to fat production by Prill *et al.* (1935), Ward *et al.* (1935) and Ikeda (1950). Hopton (pers. comm. 1956), working with yeasts, found that carbohydrate was metabolized without increased cell weight, though the pH decreased; a similar effect was observed during the present work with moulds, but the effect on the pH was comparatively small.

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A STUDY OF A GRAM-POSITIVE COCCUS RESPONSIBLE FOR ROPINESS AND VISCOSITY IN MALT WORT

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SUMMARY A Gram-positive coccus with the properties typical of pediococci produced a ropy, viscous growth in malt wort. Nutritional studies in the media of Dunn *et al.* (1947) showed pantothenic acid to be required by the organism but growth occurred in the complete medium only when a trace of malt wort was present. Seven other vitamins tested were not necessary for growth.

In a medium consisting of mineral salts, glucose and alcoholic extracts of peptone and yeast autolysate the organism grew well and the viscosity of the medium increased. Higher viscosities were attained when the buffering capacity of the medium was increased either by raising the concentrations of the alcoholic extracts or by adding sodium acetate. The material responsible for the viscosity was produced in small amounts only and appeared to be a mucopolysaccharide. The only sugar obtained on hydrolysis of this material was glucose.

Fructose, maltose, sucrose and raffinose were all fermented by the organism but only in media containing maltose was the increase in viscosity comparable with that attained in a glucose-containing medium.

Homofermentative Gram-positive cocci which form characteristic tetrad groups of cells are not uncommon contaminants of beers, beer worts and fermenting vegetables. Their taxonomy has been the subject of considerable controversy, but after a critical review of the literature and a comprehensive examination of a number of organisms isolated from vegetable fermentations, Pederson (1949) concluded that assignment to a separate genus, *Pediococcus*, was justified.

Some nine years ago the nutritional and physiological characteristics of a Gram-positive coccus, isolated from beer wort, which formed typical tetrad groups, were examined at Manchester. When cultured in beer wort, the organism exhibited a ropy growth and the medium became viscous. Diagnostic tests showed it to have properties characteristic of the pediococci. We were encouraged to present our results by the finding of Pederson, Albury & Breed (1954) that the pediococci are of value in the production of fermented cucumbers. These authors also pointed to the desirability of further studies being made on these organisms.

Materials and Methods

Preparation of constituents of media

The development of viscosity in bacterial cultures is frequently indicative of the formation of polysaccharides. Initial separation of these substances is usually achieved by precipitation of the culture fluid with alcohol. Since one aim of this work was to isolate the material responsible for viscosity, the use of culture media initially free from precipitatable organic matter offered obvious practical advantages.

Accordingly, alcoholic extracts of peptone, yeast autolysate and malt wort were prepared and examined for their ability to support growth of the organism.

Peptone extract. This was prepared by refluxing 200 g of peptone (Evans Medical Supplies, Ltd.) with four separate 500 ml amounts of ethanol, each for 1 hr. The solutions were combined, the solvent removed by distillation under reduced pressure and the extract vacuum-dried (37 g). The peptone extract was dissolved in water to a concentration of 50 g/l. and the solution sterilized. Its nitrogen content was 5·5 g/l.

Yeast autolysate extract. Brewer's yeast (1000 g) was made into a cream with 1000 ml of tap water containing ethyl acetate (1% v/v) and placed in a 30° incubator. When frothing had subsided, toluene to a 1% (v/v) concentration was included as a preservative. The yeast debris settled after several days and the dark brown supernatant was readily siphoned off. The supernatant was treated with four times its volume of absolute ethanol and the precipitate which formed was allowed to settle overnight before filtration. The ethanol was removed from the filtrate by distillation under reduced pressure, the syrup-like extract was treated with 90% (v/v) ethanol, and the settling, filtration and distillation procedures were repeated. The dried preparation (89 g) was dissolved in 500 ml of 2% (w/v) H_2SO_4 and hydrolysed to constant reducing value. Neutralization was achieved by the addition of $CaCO_3$, and the $CaSO_4$ and excess $CaCO_3$ were removed by filtration. The clear filtrate was then diluted to a concentration of 100 g/l (based on unhydrolysed extract) and sterilized. Its nitrogen content was 9·0 g/l.

Malt wort extract. For this, 2·5 l. of malt wort (S.G. 1·050) were treated in the same way as the yeast autolysate supernatant, and 86 g of extract were obtained by the ethanolic precipitation. The extract, after hydrolysis and neutralization, was diluted to a concentration of 100 g/l. and sterilized.

Methanolic extracts. In two experiments methanolic extracts of peptone and yeast autolysate were employed. The procedures for their preparation were the same as for the ethanolic extracts but the yeast extract was not hydrolysed.

Preparation and distribution of media

PYE medium. The basal medium used for quantitative experiments had the following composition: KH_2PO_4, 1·0 g; $MgSO_4.7H_2O$, 1·0 g; $FeSO_4.7H_2O$, 0·01 g; peptone extract, 5 g; yeast autolysate extract, 5 g; distilled water to 1 litre. Sugars were included at a concentration of 50 g/l. The pH after sterilization was 4·7–5·2.

Distribution. For qualitative studies of growth, completed media were dispensed aseptically in 5 ml amounts into sterile $6 \times \frac{5}{8}$ in. test tubes. For quantitative work a sterile double-strength solution of peptone extract, yeast extract and salts was mixed in the experimental flask with a double-strength sterile solution of sugar.

Inoculation, incubation and examination of cultures

For qualitative studies inocula were from 5-day cultures in malt wort. For quantitative work the inoculum was grown in PYE medium containing 50 g/l. of glucose. Inoculation was by direct transfer of the inoculum culture by loop or by pipette from

a washed cell suspension. For suspensions, the ropy mucilage was separated by centrifuging. It was washed once in saline (0·9% w/v) and then centrifuged and was finally suspended in a volume of saline equal to that of the original culture.

All cultures and controls were incubated at 25°.

Examination of a single culture at intervals was unsatisfactory for quantitative work since it was difficult to avoid withdrawing large amounts of mucilaginous rope during sampling. A series of flasks of medium was therefore inoculated and incubated, individual flasks being removed from the incubator at intervals and their contents analysed. Estimations on uninoculated controls were made periodically and it was usually found that their viscosity increased by 2–3 sec after 20 days.

Analytical methods

The analyses were made on cultures which had been filtered through Seitz pads.

Viscosity. An Ostwald viscometer was used. Viscosities are expressed as time of outflow in sec at 20°.

Glucose. The iodimetric method of Willstätter & Schudel (1918) and the semi-micro copper reduction method of Somogyi (1945) were used on different occasions.

Acid. Portions of 5 ml of culture were titrated with 0·1 N NaOH to the phenolphthalein end point.

Precipitation with alcohol. To one volume of culture (usually 100 ml) were added two volumes of ethanol or methanol and the precipitate which formed was allowed to settle overnight. After the precipitate had been collected in a sintered glass crucible it was washed with alcohol and finally dried at 130° for 60 min. The precipitates were hydrolysed with 2N H_2SO_4. The mixture, contained in a sealed tube, was heated in a boiling water bath for 4 hr. The acid was neutralized with $BaCO_3$, and the $BaSO_4$ and excess $BaCO_3$ were then filtered off. The filtrate was passed through a small ion exchange column (Zeo-Carb 215) to remove cations, and sugar in the effluent was determined by the method of Somogyi (1945).

Growth of cultures. At first visual assessments of the degree of ropiness and viscosity of the cultures were made, and expressed in terms of numbers of + signs. When quantitative estimations began to be made it became clear that the visual assessments were only very approximate, particularly with regard to viscosity.

Results

General characteristics of the organism

The organism does not grow in a simple mineral salts-glucose medium but in media such as malt wort or yeast water-glucose ropiness is produced and the medium becomes viscous and turbid. Stained preparations show the organism to be a Gram-positive coccus with tetrad groups predominant. Only very small colonies are formed on solid media in air but in carbon dioxide the colonies are larger and are predominantly entire and even. In yeast water-glucose no volatile acid is produced but inactive lactic acid is formed and the final pH value of the culture is about 3·4. Tests for catalase and acetoin are invariably negative.

Qualitative investigation of growth in various media

Growth of the organism in media containing various combinations of extracts is shown in Table 1. The conclusion that factors essential for the growth of the organism are present in malt wort but are precipitated by alcohol is supported by the finding that growth followed inoculation only in malt wort and in media prepared from precipitates obtained by addition of ethanol to malt wort. The hydrolytic treatment used in the preparation of the malt wort extract was apparently not responsible for the failure of the organism to grow. It appears that, even though the deficiency caused by alcoholic precipitation of malt wort can be partially replaced by peptone and yeast autolysate extracts, a trace of malt wort (taken over by loop inoculation) is obligatory for growth in media containing relatively low levels of these extracts.

Table 1. *The growth of the organism in media prepared from combinations of malt wort extract with peptone and yeast autolysate extracts and from malt wort and precipitates thereof obtained by fractional precipitation with alcohol*

Medium constituents	Concentration (g/l.)	Growth* after incubation for (days) with									
		Loop inoculation†					Suspension inoculation‡				
		2	5	10	15	24	2	5	10	15	24
Malt wort extract	100	0	0	0	0	0	0	0	0	0	0
Malt wort extract, unhydrolysed	100	0	0	0	0	0	0	0	0	0	0
Malt wort extract Peptone extract	100 0·5	0	±	+	+	+	0	0	0	0	0
Malt wort extract Yeast autolysate extract	100 0·5	0	0	0	0	0	0	0	0	0	0
Malt wort extract Peptone extract Yeast autolysate extract	100 0·5 0·5	0	+	+	+	+	0	0	0	0	0
Malt wort		+	++	+++	+++	+++	0	++	+++	+++	+++
Ppt. from malt wort at 70% (v/v) ethyl alcohol concentration	100	0	0	0	+	++	0	0	0	0	++
Ppt. from malt wort at 90% (v/v) ethyl alcohol concentration	100	0	0	±	++	++	0	0	0	0	++

* Visual estimation. † From a 5-day malt wort culture. ‡ Of cells washed from an 11-day culture on 5% yeast water–2% (w/v) glucose agar.

It was eventually found that a medium containing inorganic salts and 5 g/l. each of ethanolic extracts of peptone and yeast autolysate (PYE basal medium) and glucose supplied the organism's requirements and that growth in this medium initiated either by loop or suspension inoculation was comparable with that in malt wort. Later quantitative investigations showed that the maximum viscosity attained in this medium was somewhat greater than that in a medium in which ethanolic extracts were replaced by methanolic extracts.

Omission of the inorganic salts, particularly the $MgSO_4$ and $FeSO_4$, retarded growth. Some ropiness was produced in the basal medium itself but the culture did not become viscous. However, when the concentrations of the extracts were

increased threefold the ropiness which developed was comparable with that observed in malt wort cultures but there was no increase in the viscosity of the medium.

The requirements for growth factors were examined using the medium of Dunn *et al.* (1947). The eight vitamins, biotin, folic acid, *p*-aminobenzoic acid, riboflavin, inositol, pantothenic acid, nicotinic acid and thiamin, were omitted separately from the complete medium. From a loop inoculation there was good growth in all the media except the one lacking pantothenic acid. There was no growth following inoculation from a washed suspension in any of the media, which again suggested that an essential substance was transferred by loop inoculation.

Quantitative studies of culture development

A preliminary examination of a 21 day old culture in PYE-glucose medium showed that the viscosity of the culture was diminished but little by Seitz-filtration, which indicated that the viscosity is not caused by suspended cellular mucilage but is attributable to a soluble constituent. Also, on adding 2 volumes of alcohol to the Seitz-filtered culture fluid, a small amount of a gummy precipitate immediately formed and a granular precipitate settled out later. Similar treatment of the same volume of uninoculated medium resulted in the formation of a granular precipitate only. The weights of the two precipitates were very similar; but the precipitate from the culture medium, unlike that from the uninoculated medium, gave a positive Molisch test for carbohydrate, reduced Fehling's solution after hydrolysis and dissolved in water to produce a very viscous solution.

Table 2. *The results of analyses of cultures* grown in PYE-glucose medium for varying periods*

Property	Values determined on cultures incubated for (days):									Controls, after (days):		
	3	6	10	13	17	20	24	27	31	3	10	17
Viscosity (sec)												
unfiltered	291·4	309·1	317·3	347·3	322·3	336·5	315·0	320·1	314·8	287·9	288·8	290·0
Seitz-filtered	289·1	307·4	314·6	364·3	317·9	330·5	314·6	314·8	312·6	286·7	288·1	290·3
pH	4·47	4·02	3·93	3·74	3·86	3·79	3·88	3·84	3·84	4·75	4·65	4·68
Acidity (ml 0·1 N NaOH/100 ml)	23·5	31·8	36·9	52·4	39·8	47·8	38·2	45·5	45·1	23·0	21·6	22·0
Glucose (g/100 ml)	5·80	6·22	6·11	6·56	6·11	6·15	6·57	5·71	—	5·80	6·31	6·51
Weight of ppt. (mg/100 ml)	72·7	81·3	109·2	116·4	67·9	62·5	73·9	60·2	76·5	78·6	75·7	76·6
Reducing sugar as glucose (%)	—	—	—	3·57	—	—	—	—	—	—	0·59	—

* Distribution of medium, 250 ml/1500 ml flask. Inoculum, 10 ml culture/flask.

The results of a comprehensive quantitative experiment are shown in Table 2. It is seen that after an initial rise the figures for viscosity and acid production fluctuated in the same manner. This may be characteristic of the course of development of the organism or may be attributable to inequalities in the development of individual flasks of culture. The weights of precipitates from the cultures were

consistently similar to the weights of those from the controls except in two instances, but here the analyses for reducing sugar after hydrolysis showed that polysaccharide material was not responsible for the higher weight. If ropy mucilage was inadvertently introduced into the filter during the early stages of Seitz-filtration, the rate of filtration subsequently became very slow and some evaporation of filtrate undoubtedly occurred. This would also reflect on the determinations of viscosity and acidity.

Table 3. *The effect on culture development in PYE-glucose medium of doubling the concentration of basal constituents*

Property	Normal strength, after (days):					Double strength, after (days):			
	Culture				Control	Culture			Control
	10	14	18	21	15	15	18	21	15
Viscosity (sec)									
unfiltered	295·7	302·3	335·3	304·7	—	322·8	373·4	325·8	—
Seitz filtered	296·2	299·2	342·5	305·1	290·5	322·2	361·3	323·3	298·3
pH	4·23	4·12	3·74	4·02	4·79	4·15	3·73	4·00	4·83
Acidity (ml 0·1 N NaOH/100 ml)	29·8	30·6	50·8	35·6	21·2	43·4	80·8	58·6	33·6
Glucose (g/100 ml)	5·45	5·47	5·68	5·65	5·42	5·69	5·63	5·79	5·69
Weight of ppt. (mg/100 ml)	78·0	81·0	107·2	73·2	79·6	147·8	145·8	148·0	152·9
Reducing sugar as glucose (%)	—	—	6·55	—	1·30	—	4·29	—	1·30

Distribution of medium, 250 ml/1500 ml flask. Inoculum, 10 ml culture/flask.

When the development of the organism in a medium containing twice the normal concentrations of basal constituents was compared with that in normal PYE-glucose medium the results shown in Table 3 were obtained. Both viscosity and acid production were greater in the former than in the latter medium but here again there was little difference between the weights of precipitates from controls and from cultures. As in the previous experiment (Table 2) there was no clear indication of glucose utilization, a finding which may be attributable to the poor specificity of the Willstätter-Schudel method. The increase in titratable acidity indicates that some breakdown of glucose took place. In both media a longer time was taken to reach maximum viscosity than in the previous experiment (see Table 2). This again may be attributable to inequalities in the growth rates of individual cultures, but since the organism grows relatively slowly variations in the size and activity of the inocula may have been concerned.

The higher buffer capacity of the more concentrated medium may have been partly responsible for the increased development of the organism over that in the normal medium and it was shown that a high viscosity was attained in normal PYE-glucose medium buffered with 1% (w/v) sodium acetate trihydrate (Table 4). Methanolic extracts of peptone and yeast autolysate were employed and the medium was adjusted to an initial pH of 6·0. Glucose was estimated by the method of Somogyi (1945) and there was definite utilization of glucose in the buffered culture. The viscosity of the cultures in the unbuffered medium increased very little; however, as in other experiments the pH fell to a value slightly below 4·0.

Table 4. *The effect of adding acetate buffer on the development of cultures in PYE-glucose medium containing methanolic extracts of peptone and yeast autolysate*

Property	Values determined on cultures incubated for (days):						
	0	6	10	13	17	20	24
Normal medium							
Viscosity (sec)	282·6	284·5	283·2	286·7	288·6	286·3	288·7
pH	6·0	6·08	4·92	4·38	4·10	4·06	3·83
Glucose (g/100 ml)	5·00	4·90	5·10	5·00	5·00	4·96	4·89
Buffered medium							
Viscosity (sec)	289·5	290·5	291·8	303·9	332·2	337·6	340·8
pH	6·0	5·63	5·01	4·70	4·53	4·41	4·22
Glucose (g/100 ml)	5·00	4·90	4·88	4·63	4·63	4·50	4·34

Distribution of medium, 50 ml/100 ml flask. Inoculum, 1 ml culture/flask.

Analysis of precipitated material

Nine 500 ml lots of PYE-glucose medium (methanolic extracts) were inoculated each with 50 ml of culture in the same medium. At the end of 22 days, when the viscosity had increased from 294·6 to 321·5 sec, the cultures were bulked and Seitz-filtered. Culture fluid (4 l.) and uninoculated medium (400 ml) were then precipitated with 2 volumes of methanol. The precipitates were allowed to settle for 5 days and were then recovered by centrifuging. The analyses of the washed and dried precipitates are shown in Table 5. Inorganic salts are more soluble in methanol than in ethanol and consequently the weights of precipitate were less than those obtained by ethanolic precipitation. Chromatograms of the hydrolysate of the precipitate from the culture fluid, run in phenol (Partridge, 1948), showed only the presence of glucose.

Table 5. *Analyses of precipitates obtained from a 22 day PYE-glucose culture and from the uninoculated medium by treatment with two volumes of methanol*

	Precipitate from	
	4 l. of culture	400 ml of medium
Weight (mg)	348·8	27·4
Nitrogen content (%)	2·47	—
Reducing sugar content, as glucose (%)	54·2	2·19

Growth in PYE medium containing various sugars

The results in Table 6 show the behaviour of the organism in basal PYE medium containing different sugars. The highest viscosity and the lowest pH value were attained in the medium containing glucose. Maltose, sucrose, fructose and raffinose were all fermented but only the maltose cultures were appreciably viscous at 23 days. The cultures grown in media containing glucose or maltose were both ropy throughout, whereas in the medium containing sucrose the growth was powdery at first but became ropy later. Growth in the medium containing fructose was always powdery.

Table 6. *The viscosity and pH of cultures grown in PYE medium containing different carbohydrates*

Sugar		Viscosity and pH values of cultures incubated for (days):				
		0	7	14	19	23
Glucose	V*	292·4	292·5	299·1	315·6	312·8
	pH	5·17	4·36	4·02	3·82	3·89
Raffinose	V	291·7	291·6	294·8	294·2	295·7
	pH	5·17	4·83	4·73	4·73	4·76
Dextrin	V	350·0	344·1	351·3	354·8	358·9
	pH	5·17	4·99	4·98	4·97	5·05
Sucrose	V	294·4	292·5	298·1	300·4	302·1
	pH	4·99	4·57	4·17	4·19	4·21
Maltose	V	295·6	294·3	296·1	299·1	310·3
	pH	4·96	4·63	4·44	4·34	4·06
Fructose	V	289·8	293·6	292·3	293·8	295·9
	pH	4·93	4·84	4·03	4·00	4·07
Galactose	V	290·2	290·3	291·8	293·0	294·5
	pH	4·94	5·08	4·85	4·84	4·95
Arabinose	V	287·2	285·4	288·5	290·8	290·7
	pH	4·82	4·97	4·80	4·83	4·87

* V, viscosity (sec). Distribution of medium, 25 ml/100 ml flask. Inoculation, 0·2 ml suspension/flask.

Discussion

The investigations on the growth of the organism in the synthetic medium of Dunn *et al.* (1947) pointed to a requirement for pantothenic acid; but they also showed that the organism requires a factor which is not present in the complete synthetic medium but which is supplied by the trace of malt wort taken over by loop inoculation. Since this work was carried out, Jensen & Seeley (1954) have shown that pediococci, like most lactic acid bacteria, have complex growth requirements. All of 34 strains examined by these investigators required a number of amino acids and were unable to grow in the absence of pantothenic acid, niacin and 'citrovorum factor'. The latter was required in only very low concentration. The medium of Dunn *et al.* (1947) does not contain 'citrovorum factor' and it seems possible that a trace of malt wort could make up the deficiency; further studies, however, are required to establish the full nutritional pattern of the organism.

The results recorded in Tables 2 and 3 indicate that the increase in viscosity of cultures in PYE-glucose medium is paralleled by an increase in titratable acidity, a behaviour which closely resembles that observed for the formation of dextran in cultures of *Leuconostoc* species in a sucrose-containing medium (Whiteside-Carlson & Carlson, 1949). Other results (see Table 4) seem to suggest that polysaccharide formation is initiated in a particular pH range (below 5·0), a view which is supported by the finding that although the organism grew well in PYE-glucose medium containing $CaCO_3$, viscosity was not produced. The observation that far less viscosity was produced in PYE-glucose medium adjusted to pH 6 (Table 4) than in the same medium at pH 4·8 would appear to indicate that the former culture was producing acid too rapidly in the pH range of polysaccharide initiation. In a recent paper Bailey & Oxford (1958) reported that *Streptococcus bovis* produced only a little dextran

in a defined medium containing sucrose, whereas in the same medium buffered with acetate large amounts of dextran were formed; these authors also showed that lactate is a relatively poor buffer for dextran formation.

The viscosity of a culture belies the amount of polysaccharide produced, which is quantitatively very small; the rather high nitrogen content (2·47%) of the alcohol-precipitated material (Table 5) suggests that the substance responsible for high viscosity may be more of the nature of a mucopolysaccharide. Solutions of such complexes are usually very viscous (Stacey, 1946).

It seems unlikely that the presence of this organism in fermented foodstuffs could ever be considered desirable. Although it grows only slowly and does not cause pronounced chemical changes in its environment, its slimy viscous growth would spoil the physical appearance of a product.

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COLI-AEROGENES BACTERIA IN FARM WATER SUPPLIES

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SUMMARY: A series of 825 cultures of coli-aerogenes bacteria isolated at 30° and a series of 735 cultures isolated at 37° from 645 samples of farm water supplies were classified according to the recommendations of the Coliform Sub-Committee of the Society for Applied Bacteriology (Report, 1949). *Klebsiella* constituted 50% of the cultures isolated at 30°, whereas *Escherichia coli* I was the dominant type, forming 57%, among the cultures isolated at 37°. It would thus appear that isolation at 30° is as selective for *Klebsiella* as isolation at 37° is for *Escherichia*. Coli-aerogenes organisms, mainly 37° negative strains of *Citrobacter freundii* I and *K. cloacae*, were found in waters of high sanitary quality derived from protected springs and wells; but the coli-aerogenes microflora of polluted water was dominated by *E. coli* I, which formed 43% of the isolates at 30° and 76% of those at 37°. The results for a series of fortnightly samples from 11 farm water supplies showed a marked seasonal variation in the incidence of different types isolated at 30°; *E. coli* I formed a higher proportion in summer than in winter, while 37° negative strains of *Klebsiella* and *Citrobacter* formed a higher proportion in winter than in summer.

A preliminary investigation of the distribution of different types of coli-aerogenes bacteria isolated at 30° from 142 farm water supplies (Thomas, Jones & Franklin, 1951) showed that *Klebsiella* was the dominant group and that *Escherichia* was relatively common. A high proportion of these water supplies was derived from polluted surface water and unprotected springs. A survey of the incidence of different types of coli-aerogenes bacteria, isolated at 30° and at 37°, in a more representative series of 645 farm water supplies was undertaken during 1954–57, and the results are reported here.

The supplies examined included a fairly high proportion of protected springs and wells, some of which did not show the presence of coli-aerogenes organisms in 100 ml of water when examined by the MacConkey's broth test at 37°. Most of the ground waters examined were derived from Cambrian, Ordovician, Silurian and Old Red Sandstone systems and were generally of a soft, acid nature. Thus 80% of the samples gave pH readings below 7·0, with 26% below 6·0; only 1% had a pH above 8·0.

Materials and Methods

Source of samples. The work was done at ten Ministry of Agriculture laboratories in Wales, two water samples being examined every month at each laboratory. More than half the supplies examined were derived from springs which were usually sited above and some distance from the farmsteads (Table 1). One-third of the samples

were from wells, which were often sited near the dairy or farmhouse, while over one hundred samples came from surface water liable to pollution from sheep and cattle. The series is considered to be representative of the untreated water supplies intended for use for dairy purposes on farms in Wales, but it does not include any Local Authority chlorinated water, which is probably used on about one-fifth of the 27,000 milk producing farms in the Principality. Approximately 13,000 untreated farm water supplies in Wales have been examined bacteriologically at Ministry of Agriculture laboratories during the past ten years; 60% were from springs, 33% from wells, 4% upland streams, 1% boreholes, 0·5% rainwater from roofs and the remaining 1·5% from lowland surface waters.

Table 1. *Sources of the water supplies examined*

Source	No. of supplies	Proportion (%)
Borehole	3	0·5
Well	199	30·9
Spring	326	50·6
Upland stream or lake	82	12·7
Lowland stream or river	25	3·9
Canal, ditch or pond	2	0·3
Rainwater (roofs)	8	1·1
Totals	645	100·0

Samples were also taken at fortnightly intervals over a twelve month period from eleven farm water supplies, in order to determine whether there was any seasonal variation in the incidence of different types of coli-aerogenes organisms. The sources included 7 springs, 2 wells and 2 upland streams.

Sampling and testing. Each sample, consisting of about 540 ml of water, was brought to the laboratory in an insulated container and examined within 6 hr of sampling. Quintuplicate 10, 1 and 10^{-1} ml amounts of water were inoculated into MacConkey's broth and incubated for 5 days at 30°; quintuplicate sets of the same amounts were incubated for 2 days at 37° as prescribed by the Ministry of Health (Report, 1956*a*) for the sanitary examination of water supplies. The range of presumptive coli-aerogenes counts/100 ml of water at 37° is compared with those obtained

Table 2. *The relation between the presumptive coli-aerogenes contents (MPN)* at 37° and 30° of 645 water samples*

Range of MPN/100 ml (2 days, 37°)	No. of samples	Range of MPN/100 ml (5 days, 30°)			
		1–10	11–100	101–1,000	>1,000
0	66	17	31	15	3
1–10	146	21	42	59	24
11–100	232	3	46	115	68
101–1,000	128	0	4	52	72
>1,000	73	0	0	3	70
Totals	645	41	123	244	237

* MPN, most probable number.

at 30° in Table 2. A series of 66 samples did not show the presence of coli-aerogenes organisms when tested at 37°, though appreciable numbers were found in some of these samples when examined at 30°. The types of coli-aerogenes organisms isolated from these supplies are discussed separately.

All tubes positive at 37° were sub-inoculated into further tubes of MacConkey's broth incubated for 2 days at 44° to determine the presence of presumptive *Escherichia coli* I. Cultures isolated from samples giving negative *E. coli* I test results, and those derived from heavily polluted water supplies containing >250 *E. coli* I/100 ml, are also discussed separately.

Isolation and differential tests. Streaks on Difco eosin methylene blue agar were made from the highest positive dilutions at 37° and at 30°; after incubation for 1 or 2 days at 37° for the former and at 30° for the latter, one to three well isolated colonies having the characteristic appearance of coli-aerogenes bacteria were picked into peptone water. A 24 hr culture at 30° provided the inoculum for the differential tests recommended in Report (1949), incubation at 30° being used for the IMViC tests.

Results

Classification

Duplicate cultures showing identical reactions in all media were discarded, leaving 825 classifiable strains isolated at 30° and 735 isolated at 37°. The incidence of different types of these cultures is summarized in Table 3, according to the classification and nomenclature recommended in Report (1956*b*). Gram-negative rods which formed acid and gas in lactose broth within 5 days at 30° have been termed typical coli-aerogenes bacteria; this definition includes cultures which did not produce acid and gas in MacConkey's broth within 2 days at 37° and which are termed '37° negative'. Cultures which developed an acid reaction but did not produce gas in lactose broth within 5 days at 30° are termed 'anaerogenic', and Gram-negative rods isolated from characteristic coli-aerogenes colonies on eosin methylene blue agar plates but which did not produce acid or gas in 5 days in lactose broth at 30°, are considered to be 'paracolon' types.

Escherichia coli I constituted only 18% of the cultures isolated at 30°, whereas it was the dominant type among those isolated at 37°. In view of the importance attached to the 44° test for the routine examination of water in Britain it is of interest that 98% of the 564 cultures of *E. coli* I formed gas in MacConkey's broth at 44°. However, 4·6% of the 996 cultures of other types also formed gas at 44°, confirming previous results obtained by Thomas *et al.* (1955) when just over 5% of 707 cultures isolated from 44° positive tubes were types other than *E. coli* I. It is thus evident that while the 44° test is not an infallible index of the presence of *E. coli* I in water. the incidence of other 44° positive types, some of which are undoubtedly associated with human or animal faeces, is relatively infrequent in Britain.

Klebsiella was the dominant genus at 30°, embracing 50% of the cultures compared with only 17% at 37°. The much higher incidence at 30° is partly accounted for by the high proportion (50%) of the cultures which failed to form gas at 37°. This is

particularly noticeable in the case of *K. cloacae*, the dominant species at 30°, as 60% of the 242 cultures failed to form gas at 37°.

Table 3. *The types of coli-aerogenes bacteria isolated from water supplies, and their frequency*

Organism and type	Strains isolated at 30°		Strains isolated at 37°	
	No.	%	No.	%
Escherichia coli I	141		412	
44° negative	5	146 17·7	6	418 56·9
E. coli II	16		24	
44° positive	5		6	
37° negative	7	30 3·6	2	32 4·3
paracolon	2		0	
Citrobacter freundii I	68		97	
44° positive	2		8	
gelatin positive	33		3	
37° negative	25	182 22·1	1	113 15·4
37° negative, gelatin positive	20		1	
anaerogenic	6		1	
paracolon	28		2	
Cit. freundii II	24		30	
44° positive	10		8	
gelatin positive	5		3	
37° negative	7	52 6·3	1	46 6·3
37° negative, gelatin positive	1		1	
anaerogenic	2		1	
paracolon	3		2	
Klebsiella aerogenes I	79		48	
44° positive	1		4	
citrate negative	1	139 16·9	3	73 9·9
37° negative	46		14	
paracolon	12		4	
K. cloacae	88		22	
indole positive	8		5	
citrate negative	1	242 29·3	0	40 5·4
37° negative	107		9	
anaerogenic	1		1	
paracolon	37		3	
K. aerogenes II	26		10	
44° positive	1		1	
citrate negative	1	34 4·1	0	13 1·8
37° negative	4		1	
paracolon	2		1	
Totals	825	100·0	735	100·0

There was relatively little difference between the incidence of *Citrobacter freundii* I among the cultures isolated at 30° and at 37°, even though slightly more than one-third of the former did not form gas at 37°.

E. coli II and *K. aerogenes* II were both relatively infrequent, whereas the incidence of *Cit. freundii* II was somewhat higher, particularly in polluted waters, as will be shown later.

The incidence of aberrant types, e.g. 44° positive strains of *Citrobacter* and *Klebsiella*, citrate negative strains of *Klebsiella* and indole positive strains of *K. cloacae*, was not infrequent. Such aberrant or irregular combinations of IMViC and other biochemical reactions are usually encountered during the study of a large number of coli-aerogenes cultures (Stuart, Zimmerman, Baker & Rustigian, 1942; Prescott, Winslow & McCrady, 1945; Thomas, Jones & Franklin, 1951), though the stability or otherwise of these reactions does not appear to have been studied. The Enterobacteriaceae Sub-Committee (Report, 1958) described the situation as follows:—'The family Enterobacteriaceae is made up of a series of interrelated bacterial types and segregation of these into groups is often difficult because the transition from group to group is gradual, and intermediate forms exist in all instances'.

In the present investigation the occurrence of gelatin positive strains of *Citrobacter* is particularly striking. Almost one-third of the *Cit. freundii* I cultures isolated at 30° liquefied gelatin within 21 days at 22°; they resemble the *Arizona* group of Edwards, West & Bruner (1947). In addition, 12% of the *Cit. freundii* II cultures isolated at 30°, and 5% of both types isolated at 37°, were gelatin positive. Similar gelatin positive strains of *Citrobacter* have been isolated from grass and cereal crops (Thomas & McQuillin, 1952; Thomas & Hobson, 1955).

The paracolon strains of *K aerogenes* I probably represent the *Hafnia* group of Moeller (1954), but their methyl red reaction at 37° was not determined.

Table 4. *The types of coli-aerogenes bacteria forming acid and gas in lactose broth at 37°, and their frequency*

Organism and type	Strains isolated					
	30°			37°		
	No.		%	No.		%
Escherichia coli I	141	146	28·4	412	418	60·6
44° negative	5			6		
E. coli II	16	21	4·1	24	30	4·3
44° positive	5			6		
Citrobacter freundii I	68	103	20·0	97	108	15·7
44° positive	2			8		
gelatin positive	33			3		
Cit. freundii II	24	39	7·6	30	41	5·9
44° positive	10			8		
gelatin positive	5			3		
Klebsiella aerogenes I	79	81	15·7	48	55	8·0
44° positive	1			4		
citrate negative	1			3		
K. cloacae	88	97	18·8	22	27	3·9
indole positive	8			5		
citrate negative	1			0		
K. aerogenes II	26	28	5·4	10	11	1·6
44° positive	1			1		
citrate negative	1			0		
Totals		515	100·0		690	100·0

The predominance of *Klebsiella* among strains isolated at 30° as compared with *Escherichia* at 37° is only partly accounted for by the presence of large numbers of *Klebsiella* strains failing to form gas at 37°. Thus, when the distribution of the types forming acid and gas in MacConkey's broth in 2 days at 37° is summarized (Table 4), it is seen that *Klebsiella* is still dominant among cultures isolated at 30° and *Escherichia* among those isolated at 37°. It can therefore be concluded that *Klebsiella* must have multiplied more actively than *Escherichia* in MacConkey's broth at 30°, the incubation temperature having had a selective action, whereas *Escherichia* multiplied more actively than *Citrobacter* and *Klebsiella* in MacConkey's broth incubated at 37°.

Strains not forming gas in MacConkey's broth at 37°

The incidence of different types of 37° negative, anaerogenic and paracolon organisms is given in Table 5. In addition to the 310 strains isolated at 30°, a few were isolated at 37°, indicating that they survive or even multiply in MacConkey's broth at that temperature. None of the *E. coli* I strains was 37° negative, but a few 37° negative and paracolon strains of *E. coli* II occurred.

Table 5. *The types of 37° negative coli-aerogenes bacteria*

Organism and type	Strains isolated at 30°			Strains isolated at 37°		
	No.		%	No.		%
Escherichia coli I	0	0	0·0	0	0	0·0
E. coli II, 37° negative	7	9	2·9	2	2	4·45
paracolon	2			0		
Citrobacter freundii I, 37° negative	25	79	25·5	1	5	11·1
37° negative, gelatin positive	20			1		
anaerogenic	6			1		
paracolon	28			2		
Cit. freundii II, 37° negative	7	13	4·2	1	5	11·1
37° negative, gelatin positive	1			1		
anaerogenic	2			1		
paracolon	3			2		
Klebsiella aerogenes I, 37° negative	46	58	18·7	14	18	40·0
paracolon	12			4		
K. cloacae, 37° negative	107	145	46·8	9	13	28·9
anaerogenic	1			1		
paracolon	37			3		
K. aerogenes II, 37° negative	4	6	1·9	1	2	4·45
paracolon	2			1		
Totals		310	100·0		45	100·0

Klebsiella constituted 67% of the strains isolated at 30° and 73% of those isolated at 37°, while *K. cloacae* was the dominant species at 30° and *K aerogenes* I at 37°. Similar results were obtained by Stuart, Mickle & Borman (1940) during a study of what they termed 'pseudo-microaerogenic' cultures from soil, water and milk. As

many as 82% of their cultures resembled *Klebsiella*, and the remainder resembled *Cit. freundii* I in their IMViC reactions.

The majority (70%) of the 310 strains isolated at 30° were 37° negative, 3% were anaerogenic and 27% paracolon, while a similar distribution was found in the strains isolated at 37°, 67% being 37° negative, 7% anaerogenic and 26% paracolon.

Types of coli-aerogenes organisms in waters of high sanitary quality

A series of 91 cultures was isolated at 30° from 66 water samples, taken from protected springs and wells, which gave negative presumptive coli-aerogenes test results at 37° in 100 ml of water (Table 6). Though the majority were *Cit. freundii* I and *K. cloacae*, with 37° negative strains predominating, the formation of acid and gas in MacConkey's broth at 37° by 33 of the cultures and the presence of a few *E. coli* I strains indicated that these waters contained small numbers of coli-aerogenes organisms of sanitary significance. Thus *Citrobacter* and *Klebsiella*, particularly 37° negative strains probably derived from soil and vegetation, together with small numbers of *E. coli* I, appear to survive for a considerable time in ground waters of an acid nature (pH 5·0 to 6·5). Cuthbert, Panes & Hill (1955) found that faecal organisms such as *E. coli* I quickly died out in very acid soils (pH 3·0 to 4·0) but survived much longer in alkaline soils (pH 6·0 to 7·8). Our results agree in showing that *E. coli* I was rarely isolated from protected ground waters of an acid nature by the usual routine methods, but saprophytic coli-aerogenes bacteria of the genera *Citrobacter* and *Klebsiella* were not infrequently isolated at 30°.

Table 6. *The types of coli-aerogenes bacteria isolated at* 30° *from* 66 *water supplies which gave negative presumptive coli-aerogenes test results*/100 *ml at* 37°

Organism and type	Strains No.		Strains %
Escherichia coli I	7	7	7·7
E. coli II	2	2	2·2
Citrobacter freundii I	6		
gelatin positive	8		
37° negative	2	27	29·7
37° negative, gelatin positive	3		
anaerogenic	1		
paracolon	7		
Cit. freundii II			
37° negative	2	2	2·2
Klebsiella aerogenes I	1		
37° negative	10	15	16·4
paracolon	4		
K. cloacae	9		
37° negative	22	38	41·8
paracolon	7		
Totals		91	100·0

Coli-aerogenes organisms isolated from water samples giving negative E. coli *I test results at 44°*

The distribution of the types of coli-aerogenes organisms isolated from water supplies giving negative *E. coli* I test results at 44° is given in Table 7. Though relatively few strains of *E. coli* I were isolated, 42% of the 216 strains isolated at 30° and 76% of the 114 isolated at 37° formed gas in MacConkey's broth within 2 days at 37° and would therefore be taken as an indication of contamination of the water. *Cit. freundii* I and *K. cloacae* constituted 66% of the strains isolated at 30°, while *Cit. freundii* I and *K. aerogenes* I formed 64% of those isolated at 37°. These are the predominant types found on herbage and in soil and their presence probably indicates that the supplies were subject to contamination from surface water, though not necessarily from faeces.

Table 7. *The types of coli-aerogenes bacteria isolated from water supplies which gave negative* E. coli *I test results at 44°*

Organism and type	Strains isolated at 30°			Strains isolated at 37°		
	No.		%	No.		%
Escherichia coli I	10	10	4·6	10	11	9·7
44° negative	0			1		
E. coli II	2			2		
37° negative	3	6	2·8	1	3	2·6
paracolon	1			0		
Citrobacter freundii I	14			29		
44° positive	2			0		
gelatin positive	14			2		
37° negative	13	67	31·0	4	40	35·0
37° negative, gelatin positive	9			0		
anaerogenic	5			2		
paracolon	10			3		
Cit. freundii II	7			4		
44° positive	0			1		
gelatin positive	1	13	6·0	0	9	8·0
37° negative	4			3		
37° negative, gelatin positive	1			1		
Klebsiella aerogenes I	17			23		
44° positive	0			1		
citrate negative	1	39	18·1	2	33	28·9
37° negative	13			5		
paracolon	8			2		
K. cloacae	18			7		
indole positive	0			1		
citrate negative	1	76	35·2	0	12	10·5
37° negative	41			2		
paracolon	16			2		
K. aerogenes II	3			4		
citrate negative	1	5	2·3	0	6	5·3
37° negative	1			1		
paracolon	0			1		
Totals	216		100·0	114		100·0

Coli-aerogenes organisms isolated from heavily contaminated water

The distribution of different types of coli-aerogenes organisms isolated from heavily polluted water supplies, giving presumptive *E. coli* I counts at 44° of more than 250/100 ml of water, is in sharp contrast with the above results. *E. coli* I was the dominant type even at 30°, and constituted 76% of the strains isolated at 37° (Table 8).

Table 8. *The types of coli-aerogenes bacteria isolated from water supplies which gave MPN exceeding 250/100 ml at 44°*

Organism and type	Strains isolated at 30°		Strains isolated at 37°	
	No.	%	No.	%
Escherichia coli I	49		97	
44° negative	2	51 42·5	0	97 76·4
E. coli II	1		4	
44° positive	3	4 3·33	2	7 5·5
paracolon	0		1	
Citrobacter freundii I	5		4	
44° positive	0		2	
gelatin positive	2	11 9·2	0	10 7·9
37° negative	1		2	
paracolon	3		2	
Cit. freundii II	5		3	
44° positive	5		2	
gelatin positive	2		0	
37° negative	1	15 12·5	0	6 4·7
anaerogenic	1		0	
paracolon	1		1	
Klebsiella aerogenes I	11		4	
citrate negative	1		0	
37° negative	3	16 13·33	0	5 3·9
paracolon	1		1	
K. cloacae	6		2	
indole positive	3		0	
citrate negative	1	19 15·8	0	2 1·6
37° negative	6		0	
paracolon	3		0	
K. aerogenes II	3		0	
44° positive	1	4 3·33	0	0 0·0
Totals	120	100·0	127	100·0

Klebsiella constituted only 33% of the strains isolated at 30° and 5% of those isolated at 37° from polluted waters, whereas it formed a high proportion of the strains from waters of high sanitary quality (Tables 6 and 7). Similar results were obtained at 30° by Lewis & Pittman (1928), only 26% of the 83 strains from polluted waters being citrate positive, i.e. resembling *Klebsiella* or *Citrobacter*, whereas 66% of the 237 strains of coli-aerogenes bacteria from unpolluted waters were citrate positive. The incidence of cultures which failed to form gas in MacConkey's broth at 37° was also very low in polluted waters, being only 17% of the cultures isolated at 30° compared with 64% of the cultures from protected water supplies (Table 6).

Cit. freundii II formed as much as 12% of the strains isolated at 30°. This organism is rarely found on herbage and cereals and is considered to be of infrequent occurrence in faeces. It is thus likely that its presence in polluted water is due to surface washings from soil. One-third of the 21 *Cit. freundii* II cultures were 44° positive. Thomas, Jones & Franklin (1951) had previously found that 5 of 11 cultures of this type isolated from polluted farm water supplies formed gas in MacConkey's broth at 44°. It differs from *E. coli* I only in being citrate positive. Previous investigators have recorded very few 44° positive strains of this type in water, but they used incubation at 37° for the citrate test. The higher incidence found in the present investigation may have been due to carrying out the citrate test at 30° and thus obtaining growth when otherwise the cultures would be regarded as *E. coli* I.

The difference between the frequency distribution of the predominating types of coli-aerogenes organisms isolated from waters of high sanitary quality (giving negative *E. coli* I test results at 44°) and from heavily polluted waters (containing >250 presumptive *E. coli* I/100 ml) is much more marked at 37° than at 30° (Table 9). This becomes even more evident when the ratio of the percentage incidence of *E. coli* I to *Klebsiella* in water of good quality is divided by the ratio for polluted water. Thus at 30°, with ratios for good and polluted water of 11·0 and 0·7 respectively, the figure of 15·7 is obtained; whereas at 37°, with corresponding ratios of 4·4 and 0·08, the figure is 55·0.

Table 9. *A comparison of the incidence of different types of coli-aerogenes bacteria isolated from water supplies of high sanitary quality and from heavily polluted water*

Organism and type	Percentages of strains isolated at			
	30° from		37° from	
	Unpolluted* waters	Polluted† waters	Unpolluted waters	Polluted waters
Escherichia coli I	5	43	10	76
E. coli II	3	3	3	5
Citrobacter freundii I	31	9	35	8
Cit. freundii II	6	13	8	5
Klebsiella aerogenes I	18	13	29	4
K. cloacae	35 } 55	16 } 32	10 } 44	2 } 6
K. aerogenes II	2	3	5	0
Totals	100	100	100	100
Numbers of strains	216	120	114	127

* Waters giving negative *E. coli* I test results/100 ml at 44°.

† Waters containing >250 *E. coli* I/100 ml.

Another distinctive difference between waters of high sanitary quality and polluted waters is the remarkably high proportion of 37° negative, anaerogenic and paracolon strains found in the former (64%, Table 6 and 58%, Table 7) and their relative infrequency (17%) in the latter.

Seasonal incidence

The frequency distribution of the different types of coli-aerogenes bacteria isolated at 30° from fortnightly samples taken from each of eleven farm water supplies is given in Table 10. There was a much higher proportion of *E. coli* I strains during summer (May–October) than during winter (November–April), and this was associated with higher coli-aerogenes and *E. coli* I counts in summer. Thus 53% of the 324 samples examined during the summer six months contained more than 50 coli-aerogenes organisms/100 ml, as determined by the 37° test, compared with 38% of the 323 samples examined during the winter six months. Further, 58% of the summer samples contained more than 5 presumptive *E. coli* I/100 ml as determined by the MacConkey's broth test at 44°, compared with 29% of the winter samples.

The routine results for the main series of 645 water samples also showed seasonal variation. Coli-aerogenes counts at 37° of more than 50/100 ml were recorded for 47% of the 178 mid-summer (June–August) samples compared with 31% of the 126 mid-winter (December–February) samples. The *E. coli* I counts at 44° exceeded 10/100 ml in 60% of the former and 41% of the latter. However, the coli-aerogenes counts at 30° showed less seasonal variation, 82% of the mid-summer samples and 77% of the mid-winter samples giving most probable numbers of more than 50/100 ml of water. These results agree with previously reported increases during summer in the coli-aerogenes organisms content of farm water supplies (Thomas, Jones & Hobson, 1949; Cuthbert, 1954, 1955), and of water from watercress beds (Baker & Billing, 1958).

Table 10. *The effect of season on the types of coli-aerogenes bacteria isolated at* 30° *from samples taken at fortnightly intervals from* 11 *farm water supplies*

Organism and type	Winter		Summer	
	No.	%	No.	%
Escherichia coli I	37	8·9	83	29·3
E. coli II	14	3·4	7	2·5
Citrobacter freundii I	123	29·8	70	24·7
Cit. freundii II	15	3·6	23	8·1
Klebsiella aerogenes I	42	10·2	22	7·8
K. cloacae	178	43·1	73	25·8
K. aerogenes II	4	1·0	5	1·8
Totals	413	100·0	283	100·0

A quite marked seasonal variation was also observed in the incidence of 37° negative, anaerogenic and paracolon strains, which together formed 46% of the cultures isolated during summer and as much as 70% of those isolated during winter. Thus the coli-aerogenes microflora of the eleven water supplies had a higher proportion of *E. coli* I and a lower proportion of non-lactose-fermenting strains (at 37°) of *Klebsiella* and *Citrobacter* during the summer six months than during the winter six months. A larger seasonal difference in the proportion of *E. coli* I would probably have been shown in cultures isolated at 37° and if cultures isolated during mid-summer

(June–August) were compared with those isolated during mid-winter (December–February) with this series, but this, unfortunately, was not done.

While these results contribute to the information on seasonal variation in the incidence of different types of coli-aerogenes organisms in water, they do not indicate whether the increase in *E. coli* I in summer is due to the higher temperature of the soil and ground water, to the heavy August rainfall or to the fact that cattle graze during the summer six months. Cuthbert (1954) stated that the combination of these three seasonal factors undoubtedly has an influence on the seasonal variation.

Discussion

The predominance of *Klebsiella*, the frequent occurrence of *Citrobacter* and the relative infrequency of *Escherichia coli* I among coli-aerogenes isolates at 30° from untreated farm water supplies is in general agreement with the results of the classical investigations of Rogers, Clark & Evans (1915) on grain and green oats and of Koser (1924) on unpolluted soil.

It is recognized that the distribution of different types of coli-aerogenes bacteria isolated at 37° from natural habitats results in the preferential isolation of *E. coli* I; but it has been claimed that isolation at 30° is more likely to represent the actual proportions of the different types found under natural conditions. However, the present results indicate that isolation at 30° is as selective for *Klebsiella* as is isolation at 37° for *Escherichia*. It is therefore apparent that an ecological study of the coli-aerogenes microflora of any habitat should include isolation at 30° as well as at 37° as recommended by the Coliform Sub-Committee of the Society for Applied Bacteriology (Report, 1949).

Where the sanitary significance of a material is being determined, it may be essential that coli-aerogenes tests be carried out at 37° and 44°, as it is possible that at 30° certain types of no sanitary significance could outgrow those indicative of faecal pollution. Thus the formation of acid and gas in MacConkey's broth at 37° and at 44° is retained as the essential criterion for the determination of the coli-aerogenes and *E. coli* I contents respectively in the assessment of the sanitary quality of water in Britain (Report, 1956*a*). However, incubation at 30° is used in England and Wales for the routine enumeration of coli-aerogenes bacteria in milk and milk products, where 'low temperature' strains of *Klebsiella* and *Citrobacter* are among the most active spoilage organisms (Thomas, 1955; Thomas, Hobson & Elson, 1958).

Only a limited number of biochemical tests were applied to the large series of cultures examined during the present investigation, but it is considered that they sufficed to indicate the main ecological groups. For a detailed study of the taxonomy and nomenclature of the organisms it would be desirable to use the additional biochemical tests recommended by Kauffmann (1954), Edwards & Ewing (1955), the Enterobacteriaceae Sub-Committee (Report, 1958) and Hormaeche & Edwards (1958). In future investigations of this kind it should be possible to select at random a smaller but representative series of cultures from the isolates and to apply the complete set of tests to them. A taxonomist's view of the classification and nomenclature utilized in the present investigation, i.e. that recommended by the Coli-

Aerogenes (1956) Sub-Committee of the Society for Applied Bacteriology (Report, 1956*b*), has been given by Cowan (1956). It is pertinent to observe that the 'Enterobacteriaceae group of investigators' consists mainly of medical and veterinary bacteriologists who have been primarily concerned with cultures derived from intestinal sources or from diseases of man and domestic animals, whereas the Coli-Aerogenes (1956) Sub-Committee and Cowan (1956) have in the main considered descriptive names and abbreviations likely to be useful to applied bacteriologists dealing with coli-aerogenes bacteria found in water, milk and milk products. The need for applying the standard tests recommended by the Enterobacteriaceae Sub-Committee (Report, 1958) to cultures isolated from natural habitats such as soil, water, cereals, grass, hay and silage is becoming very evident; the results would form a valuable contribution to the taxonomy of organisms classified in the *Citrobacter* and *Klebsiella* groups.

Further information is required on the serological classification of *E. coli* I cultures derived from polluted water, according to the antigenic scheme established by Kauffmann (1954), as the epidemiological evidence and the correlation between serotypes and outbreaks leaves no doubt that certain forms of infantile gastro-enteritis must be due to specific types. Thomson (1956) found that about 1% of 1,316 samples of farm milk supplies examined in South Wales contained strains of *E. coli* I of the O groups associated with infantile gastro-enteritis, while 13% of 270 samples of poultry droppings contained similar organisms. It is probable that the milk had become infected from polluted water used for washing the dairy utensils. More recently, Jones (1958, pers. comm.) has isolated serotypes of *E. coli* I potentially pathogenic for man from heavily contaminated farm water supplies in Cambridgeshire.

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THE BACTERIAL CONTENT OF FARM DAIRY EQUIPMENT

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SUMMARY: The results obtained during the examination of 2,310 advisory rinses of farm dairy equipment by means of colony count on Yeastrel milk agar at 30°, coli-aerogenes test at 30° and milk spoilage organisms (MSO) test at 22°, are discussed in relation to attainment of proposed satisfactory colony count levels. A much higher proportion of rinses of milking machine clusters gave unsatisfactory results than those of metal equipment. The MSO test (3 days, 22°) was a more sensitive indicator of the presence of milk spoilage organisms than the coli-aerogenes test, and is recommended for routine use. Results for milking machine clusters sterilized with steam were much better than those for clusters claimed to have been cleansed by chemical methods.

Rinses and swabs have been used for assessing the bacteriological condition of milk plant and equipment for several years. The techniques which are now commonplace practice (Ministry of Agriculture, 1942, 1943) were drawn up under emergency conditions during the last war when it became necessary to maintain a high standard of plant cleansing efficiency in order to avoid wastage of milk. Bacteriologists from all sections of the industry pooled their knowledge and proposed standard methods for use in all control laboratories. The techniques describe the examination of milk cans by means of rinses, and of other equipment, which could not be readily rinsed, by means of the swab method. Both the methods and bacteriological levels suggested for colony counts at 37° proved, in practice, quite satisfactory for the purpose intended and brought about a marked improvement in the general level of plant hygiene throughout the country.

The rinse method was also extensively used in connection with advisory work at farms, but it suffered from the disadvantage that the equipment used there varied considerably, both in type and capacity, from that used at creameries. Because of this some standardization of the method of rinsing, and of interpreting the results according to varying bacteriological levels, became desirable. To illustrate the wide differences in areas rinsed, while a milking machine cluster represents an area of 1 ft², a 10 gal milk can represents 7 ft² and an 80 gal milk cooler 10 ft². Further, work on thermoduric colony counts by Thomas *et al.* (1950) had shown that colony counts done at 30° instead of at 37° gave a much more satisfactory estimation of the bacteriological condition of dairy plant.

These problems were very fully investigated by Advisory Bacteriologists of the National Agricultural Advisory Service and their recommendations were embodied in two techniques (Ministry of Agriculture, Fisheries & Food, 1955*a*, 1955*b*). In addition to revised techniques, satisfactory bacteriological levels at 30°, based on unit

area of equipment rinsed, were suggested. The proposals were: $<10^4$ colonies/ft^2—satisfactory; 10^4–5×10^4 colonies/ft^2—could be improved; $>5\times10^4$ colonies/ft^2—unsatisfactory. These recommendations have been in use for over 2 years and the time is opportune to review their value and effect upon the results obtained with farm dairy equipment. The results discussed in this paper were obtained during 1956–57 at 14 Ministry of Agriculture laboratories in Wales with rinses taken during the course of advisory work at dairy farms in the Principality.

Methods

Rinse samples. These were examined within 4 hr of rinsing. Each rinse comprised 500 ml of quarter-strength Ringer's solution. When used for rinsing hypochlorite-treated equipment it contained 0·05% (w/v) of sodium thiosulphate; 5 ml of a 4% (w/v) lecithin–6% (w/v) Lubrol W solution were added immediately after rinsing equipment cleansed with quaternary ammonium compounds.

Colony count at 30°. This was determined by plating 1 ml of the rinse and 1 ml of a 10^{-1} dilution on Yeastrel milk agar (YMA), with incubation for 3 days at 30°.

Coli-aerogenes test at 30°. Three tubes of MacConkey's broth were each inoculated with 1 ml of rinse and incubated for 3 days at 30°. The presence of acid and gas in two or more tubes was recorded as positive.

Milk spoilage organisms (MSO) test at 22°. One ml of rinse was inoculated into 5 ml of sterile litmus milk and incubated at 22°. Observations were made after 2 and 3 days, any reaction being recorded as positive.

Results

The results obtained with 2,310 rinses taken at approximately 750 farms are given in Table 1, the data being given separately for rubber and metal equipment. It is generally recognized that milking machine clusters are the most difficult pieces of dairy equipment to cleanse. Much better results were obtained throughout with rinses of metal equipment, 36% attaining the satisfactory level proposed, compared with only 22% in the case of rubber parts. About two-thirds of the milking machine clusters gave unsatisfactory colony counts, over 5×10^4 colonies/ft^2, compared with one-half in respect of metal parts, while as many as 40% of the clusters were grossly contaminated ($>2{\cdot}5\times10^6$ colonies/cluster).

Experience has shown that when the colony counts are in excess of $2{\cdot}5\times10^5$/cluster the milk drawn through them will often be of poor keeping quality. In order to study the possible effect of the bacteriological condition of the clusters on the milk, tests were also made to assess the incidence of coli-aerogenes and milk spoilage organisms in the rinse solutions. These additional tests are particularly valuable and are recommended for advisory purposes because they distinguish organisms capable of causing milk spoilage from those which do not, and the results are considered in conjunction with the total rinse count. This enables more weight to be given where there is evidence of bacteria capable of causing active milk spoilage, irrespective of the total colony count level.

Table 1. *A comparison of the distribution of colony counts of rinses of milking machine clusters and of metal equipment*

Range of colony count YMA, 3 days, 30°	Rinses of			
	Milking machine clusters		Metal equipment	
(10^3/ft²)	No.	%	No.	%
<5	190	16·4	329	28·5
5– 10	74	6·4	89	7·7
>10– 25	69	6·0	74	6·4
>25– 50	58	5·0	85	7·4
>50– 250	146	12·6	186	16·2
>250–2,500	151	13·1	225	19·5
>2,500	469	40·5	165	14·3
Totals	1,157	100·0	1,153	100·0

The results of these additional tests have been summarized separately for milking machine clusters and metal equipment in Tables 2 and 3 respectively. It will be observed (Table 2) that coli-aerogenes and milk spoilage organisms were rarely found in rinses which gave colony counts within the satisfactory level of under 10^4/cluster, while most of the clusters with unsatisfactory colony counts showed the presence of both types of organisms. Over 56% of the clusters which were grossly contaminated showed the presence of coli-aerogenes bacteria and as many as 84% the presence of milk spoilage organisms. Very similar results were recorded (Table 3) with the rinses of metal equipment, and it is evident that the MSO test is more sensitive than the coli-aerogenes test. Because of this there is much to recommend it as an additional test to the total colony count for the demonstration of the possible effects on the milk supply of unsatisfactory methods of cleansing milking equipment.

Table 2. *The incidence of coli-aerogenes and milk spoilage organisms in rinses of milking machine clusters*

Range of colony count YMA, 3 days, 30°	No. of rinses	Positive tests for					
		Coli-aerogenes organisms		Milk spoilage organisms			
				2 days		3 days	
(10^3/ft²)		No.	%	No.	%	No.	%
<5	190	0	—	4	2·1	10	5·3
5– 10	74	2	2·7	2	2·7	6	8·1
>10– 25	69	4	5·8	9	13·0	19	27·5
>25– 50	58	4	6·9	10	17·2	12	20·7
>50– 250	146	24	16·4	35	24·0	66	45·2
>250–2,500	151	61	40·4	63	41·7	101	66·9
>2,500	469	266	56·7	338	72·1	395	84·2
Total rinses	1,157	361	31·2	461	39·8	609	52·6

The opportunity was taken during the investigation to compare the value of the MSO test when the fermentation of litmus milk at 22° is recorded after 2 instead of 3 days. It is evident from Tables 2 and 3 that valuable information would be lost by

making observations after incubation for only 2 days. Here, the overall losses would have been 13% with the milking machine clusters and 9% with the metal equipment. As expected, the differences were more marked with rinses having high total colony counts.

Table 3. *The incidence of coli-aerogenes and milk spoilage organisms in rinses of metal dairy equipment*

Range of colony count YMA, 3 days, 30°	No. of rinses	Positive tests for					
		Coli-aerogenes organisms		Milk spoilage organisms			
				2 days		3 days	
(10^3/ft^2)		No.	%	No.	%	No.	%
<5	329	6	1·8	14	4·2	18	5·5
5– 10	89	9	10·1	12	13·5	18	20·2
>10– 25	74	7	9·4	17	23·0	22	29·7
>25– 50	85	28	32·9	26	30·6	37	43·5
>50– 250	186	70	37·6	69	37·1	98	52·7
>250–2,500	225	104	46·2	123	54·7	151	67·1
>2,500	165	92	55·7	107	64·5	129	78·2
Totals	1,153	316	27·4	368	31·6	473	41·0

A study was also made of the rinse results obtained with milking machine clusters which had been cleansed either with steam, hypochlorite or quaternary ammonium compounds, or wet stored in caustic soda. The latter should not be confused with immersion cleaning; the clusters were simply wet stored in a 0·5% (w/v) solution of caustic soda between milkings.

Table 4. *The incidence of different ranges of colony count among rinses of variously cleansed milking machine clusters*

Range of colony count YMA, 3 days, 30°	Method of cleansing							
	Steam		Detergent-sterilizer: hypochlorite		Detergent-sterilizer: QAC		Wet storage in caustic soda	
(10^3/ft^2)	No.	%	No.	%	No.	%	No.	%
<10	240	76·0	93	30·1	20	18·9	29	22·5
>10– 50	49	15·5	49	15·8	17	16·0	33	25·5
>50–2,500	26	8·2	79	25·6	38	35·8	50	38·8
>2,500	1	0·3	88	28·5	31	29·3	17	13·2
Totals	316	100·0	309	100·0	106	100·0	129	100·0

The results for steam sterilized equipment (Table 4) were excellent, 76% of the rinses giving colony counts of $<10^4$/cluster. They thus serve as a guide to the standard which can be attained when a proper cleansing routine is consistently applied. By comparison, the results for all the other methods were poor. The results obtained at farms claiming to be using sodium hypochlorite were, on the whole, better than those where quaternary ammonium compounds or wet storage in caustic

soda were stated to be used; 30% of the rinse samples taken at these farms were satisfactory, compared with only 22% in the case of caustic soda and 19% with quaternary ammonium compounds.

Discussion

A high proportion of rinses of efficiently cleansed farm dairy equipment can be expected to give a colony count at 30° of $<10^4/ft^2$. This is not too high a standard to attain since, in the case of creamery plant, colony counts at 30° of over $5\times10^3/ft^2$ would be considered poor. As many as 76% of the rinses of milking machine clusters sterilized by steam had colony counts under $10^4/ft^2$ and only 8% had counts exceeding $5\times10^4/ft^2$. Bearing this in mind, a remarkably high proportion of the advisory rinses of milking machine clusters exceeded this level, confirming that unsterile dairy equipment is common on farms where milk has been rejected or has failed the statutory test.

Milking machine clusters containing $>2{\cdot}5\times10^6$ viable organisms, including coli-aerogenes bacteria and active milk spoilage organisms, are considered to be grossly contaminated. Colony counts of this magnitude were very common with advisory rinses of milking machine clusters, whereas they were relatively infrequent with metal equipment. The rôle of the poorly cleansed cluster as a potential source of milk spoilage is again emphasized and the results obtained with rinses of metal equipment and with milking machine clusters indicate that the bacteriological levels suggested are reasonable provided regular attention is paid to the daily cleansing routine.

The comparative results obtained for equipment sterilized by steam and the various chemical methods should not be interpreted as an indication of their respective merits, for the majority were obtained at farms where poor keeping quality milk had recently been reported and only rarely was it found that approved methods of applying chemical sterilization were being followed. Hobson *et al.* (1958), during a two year bacteriological survey of various cleansing methods used under approved conditions, found that the results for farms using hypochlorite sterilization were not in any way inferior to those obtained at farms using steam or boiling water.

Deviations from approved methods of chemical sterilization included the intermittent use of detergent-sterilizer, i.e. two or three times a week only, the use of cold detergent-sterilizer solutions much below the recommended strength, and the use of old and sometimes cracked milking machine rubbers together with failure to de-fat them. Where wet storage was being used it was frequently found that far too much reliance was being placed on this treatment, with consequent neglect of the basic cleansing process. The build-up of milk stone on metal equipment to such an extent as to be visually obvious was relatively uncommon and the application of milk stone remover was necessary to show its presence.

The additional information obtained by the use of the coli-aerogenes or MSO tests is of such great practical value for advisory purposes that we recommend that one or other of them should be applied regularly to rinses of farm dairy equipment. Both are simple, cheap, easily set up and do not present undue difficulties in the interpretation of the results. In survey work or studies of a more investigational nature,

such as comparing methods of cleansing equipment, both should be used as a matter of course.

The MSO test gave quite informative results after 2 days incubation but a much higher proportion of positive results was obtained after 3 days. Since the total colony count is not readily available in 2 days we suggest the use of a 3 day incubation period for the MSO test, though earlier inspection may be valuable in specially urgent cases in order to demonstrate to the milk producer that his methods of cleansing dairy equipment are unsatisfactory.

Grateful acknowledgement is made to the staff of the NAAS and NMTS laboratories who participated in this investigation.

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THE MICROBIOLOGICAL EXAMINATION OF BUTTER

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SUMMARY: Butter made on farms and at creameries was examined microbiologically within 24 hr of production. The overall care exercised in the manufacture of the butter was reflected in the results obtained with the various tests used. Colony counts of psychrophiles, coli-aerogenes organisms, and yeasts and moulds were the most useful and economical tests for providing information on the standard of hygiene during manufacture, and they gave some indication of the potential keeping quality of the butter. The following satisfactory levels are recommended for creamery butter: psychrophiles $<10^3$/g, coli-aerogenes organisms <10/g and yeasts and moulds <50/g.

Examination for texture, colour and flavour has been the traditional method of grading farm butter for a very long time. Coincident with the development of creamery butter making and the introduction of cream pasteurization a number of microbiological tests came into use at creamery and control laboratories, particularly in the exporting Dominion countries and in the United States. Creameries where large amounts of butter were being made, as well as the control laboratories of some of the butter buying concerns in Britain, have also carried out microbiological examinations of butter.

Tentative standard methods have been recommended by the American Butter Institute (1937), the New Zealand Department of Agriculture (Moir & Russell, 1939), the British Standards Institution (Specification, 1940), the American Public Health Association (1941) and the West Australia Department of Agriculture (Cullity & Needham, 1947). Some of these include standards for the various tests used and they agree on the desirability of attaining the virtual absence of yeasts, moulds and coli-aerogenes bacteria, and the minimum number of lipolytic organisms.

The present report deals with an investigation into the suitability of a number of microbiological tests designed to determine the efficiency of the pasteurization of cream, the degree of post-pasteurization contamination of cream and the presence of various types of spoilage organisms in creamery butter.

Materials and Methods

Farm butter made from raw cream, and creamery butter made from pasteurized and from vacreated cream were examined within 24 hr of manufacture.

The samples were prepared for examination according to the technique described by the British Standards Institution (Specification, 1940), and nine examinations were carried out. (1) The mesophilic colony count on Yeastrel milk agar (YMA) incubated for 5 days at 30°; (2) the psychrophilic colony count on YMA incubated for 14 days at 3–5°; (3) the thermoduric colony count on YMA incubated for 5 days at 30°, after laboratory pasteurization at 65° for 35 min of 2 ml of melted butter in 8 ml of sterile skim milk; (4) the caseolytic colony count on 30% (v/v) milk agar

incubated for 5 days at 30° (Smith, Gordon & Clark, 1952); (5) the lipolytic colony count after 5 days at 22° on tributyrin agar (Specification, 1940), and on triolein agar (El Sadek & Richards, 1955); (6) the coli-aerogenes colony count on violet red bile agar (VRB) incubated for 20–24 hr at 30° (Druce *et al.* 1957); (7) the yeast and mould colony count on wort agar acidified to pH 3·5 and incubated for 5 days at 22° (Specification, 1940); (8) the detection of *Pseudomonas putrefaciens* by enrichment in litmus milk at 3·5° (Corley, Long & Hammer, 1943) with subsequent streaking on gelatin iron agar (Thompson, 1950), and (9) an examination of the texture, flavour and aroma of the unmelted butter in a glass container after holding for 7 days at 22°.

Results

A series of 56 butters of various kinds was examined by these tests and the results for 10 selected samples, which represent the types of result obtained with butter manufactured under different conditions, are given in Table 1. They range from heavily contaminated farm butter containing large numbers of spoilage organisms of all kinds to creamery butter manufactured under excellent conditions from vacreated cream so as to contain the minimum number of spoilage organisms.

Table 1. *The numbers of various kinds of organism (/g) in samples representing different types of butter and conditions of manufacture*

Sample no.	Type of butter	Meso-philes 5 days, 30°	Psychro-philes 14 days, 3–5°	Thermo-duric organisms 5 days, 30°	Caseo-lytic organisms 5 days, 30°	Lipolytic organisms 5 days, 22° on		Coli-aerogenes organisms VRB agar, 20–24 hr, 30°	Yeasts and and moulds Wort agar, 5 days, 22°
						Tri-butyrin	Tri-olein		
1	Farm butter	1,460,000	758,000	Not done	2,000	11,000	80	10	38,500
2	Farm butter; polluted water used	21,000	15,000	1,650	2,000	10,000	3,500	9,500	190
3	Farm butter chlorinated water used	20	10	450	20	<10	<10	24	10
4	Creamery butter; polluted water used	>500,000	200,000	2,050	3,000	30,000	10,000	820	20
5	Creamery butter; polluted water used	135,000	7,700	90,500	2,600	31,000	1,000	86	280
6	Creamery butter; chlorinated water used	6,100	<10	815	700	10	10	0	10
7	Creamery butter; contaminated cream	97,000	13,600	>25,000	14,000	53,000	60	13	2,340
8	Creamery butter; contaminated cream	>500,000	675,000	270	60,000	98,000	26,000	140	10
9	Creamery butter; good conditions	6,600	320	1,350	<10	10	10	0	10
10	Creamery butter; good conditions	3,200	<10	210	80	1,600	10	1	10

The farm butter (sample 1, Table 1) was made from ripened raw cream and the water used for thinning the cream, tempering the equipment and washing the butter grains was contaminated, while the butter churn and wooden worker had only been

rinsed in scalding water. The butter contained large numbers of mesophilic and psychrophilic organisms. Many of these hydrolysed tributyrin, but surprisingly very few hydrolysed triolein, while the caseolytic colony count was not abnormally high. This butter was heavily salted, and the unexpectedly low content of coli-aerogenes organisms might be attributable to this. The yeast and mould count was extraordinarily high, due in all probability to the use of an old, unsterile wooden churn and worker. *Pseudomonas putrefaciens* was present in this butter along with fluorescent pseudomonads and it became rancid within a few days at 22°.

The effect of heavily contaminated water on the microflora of unsalted butter made under farm conditions from pasteurized cream, and the resulting improvement when chlorinated water was used at this farm are illustrated by the results for samples 2 and 3. The former showed the presence of large numbers of lipolytic psychrophiles together with an exceptionally high coli-aerogenes content. The treatment of the wash water so as to give a residual of 5 p/m of chlorine after an 18 hr contact time resulted in the destruction of most of the spoilage organisms. Where contaminated water had been used the butter became rancid in 7 days, while chlorination of the water resulted in an untainted butter sample after 14 days at 22°.

Samples 4, 5 and 6 were from a creamery where normally the water supply was efficiently chlorinated, but occasionally failure to do this resulted in heavily contaminated water being used in the butter dairy. As the cream was being efficiently heat treated during this period the effect on the butter of contamination by the water was very marked. Mesophilic and psychrophilic spoilage organisms with strongly lipolytic properties, together with large numbers of coli-aerogenes organisms, were present when the water was not being chlorinated properly, whereas they were absent or present in very small numbers only when well chlorinated water was being used. The use of contaminated water resulted in the rapid development of a rancid taint in the butter, whereas no taint appeared in 14 days when the water had been properly chlorinated.

On some occasions, after overnight storage at 40°F at one creamery (sample 7) and at 50°F at another (sample 8), the heat-treated cream contained a luxuriant flora of varied types including psychrophilic and lipolytic organisms, coli-aerogenes bacteria, and yeasts and moulds. This was brought about by post-pasteurization contamination from the pipe lines, cocks and storage vats. The cream was being held at 50°F for nearly 48 hr at the second creamery; consequently mesophilic bacteria, such as coli-aerogenes organisms, and psychrophilic pseudomonads multiplied rapidly (sample 8).

Where butter was manufactured under good conditions in efficiently sterilized plant so as to give the minimum amount of post-pasteurization contamination, and where the water was efficiently chlorinated, the psychrophilic, caseolytic, lipolytic, and yeast and mould colony counts often did not exceed 10/g, while coli-aerogenes organisms were generally absent in 1 g (samples 9 and 10).

Discussion

Although in this investigation it was necessary to use many microbiological tests, it is obviously desirable in a butter-manufacturing dairy to use a few tests which

will give early warning of faults in manufacture. The following tests are suggested: a psychrophilic colony count at 3–5°, a yeast and mould count, and determination of the coli-aerogenes content on VRB agar. The psychrophilic colony count obtained on YMA incubated for 7 days at 3–5° provides information on the total number of Gram-negative spoilage organisms and, since lactic streptococci will not grow at 3–5°, a colony count at this temperature is more useful than one at 30°. Wort agar acidified to pH 3·5 and incubated for 5 days at 22° as recommended by the British Standards Institution (Specification, 1940) will give the yeast and mould content; the presence of these organisms in large numbers indicates aerial contamination and unsterile butter churns. While these two tests provide valuable information in about 7 days, the coli-aerogenes colony count on VRB agar is available within 24 hr and these organisms, being heat- and chlorine-labile, afford a measure of post-pasteurization and water contamination.

The final arbiter, however, of microbiological quality in butter must be its keeping quality, as judged by flavour and texture. Various tests are described by McDowall (1953), but the most useful appears to be the 8 day incubation test. In this, 3 oz of butter are stored in a glass jar for 8 days at 70°F and then held at 50°F for 12–14 hr before being graded. This is a compulsory test in the United States Federal Examination for the grading of Certified Butter. Observations made during the present investigation confirmed that this incubation test gives an early indication of the imminence of taints, such as those produced by pseudomonads.

Early in the present investigation an attempt was made to reduce the number of tests by using the double indicator agar (DIA) described by Donovan & Vincent (1955) to obtain both the mesophilic and the caseolytic colony counts from one plating. The results were not altogether satisfactory as it was extremely difficult to detect caseolysis on the highly coloured agar. This was also the experience of Donovan & Vincent, and a change was made, therefore, to the 30% milk agar of Smith, Gordon & Clark (1952).

The results have shown the value of using a chlorinated water supply; a chlorination dosage of 5–10 p/m should normally prove sufficient except where the organic matter content of the water is very high. In view of the importance in butter manufacture of a pure water, the supply should be examined regularly at the creamery laboratory. The tests should include the residual chlorine content, the psychrophilic and lipolytic colony counts and tests for coli-aerogenes organisms. It is possible that the psychrophilic and lipolytic colony counts might be combined by incubating tributyrin or triolein agar for 14 days at 3–5°. Incubation of MacConkey's broth for 7 days at 22° will detect contaminants likely to cause butter deterioration under normal storage conditions. A positive result in MacConkey's broth incubated for 2 days at 37° would have sanitary significance and indicate that the butter might have become contaminated with pathogenic organisms.

Under modern conditions of butter manufacture, where the cream is pasteurized and vacreated and the rinse water is of a high degree of purity, butter can be produced which contains few organisms, and virtually no spoilage types. Consequently, high standards can be set in the various tests with a reasonable likelihood that most samples will comply with them. The standards used in the butter exporting countries,

together with the results obtained during the present investigation, provided a basis for recommending that: (1) The psychrophilic colony count (14 days at 3–5°) should not exceed 10^3/g. The psychrophiles are heat-labile Gram-negative rods, indicative of post-pasteurization contamination. (2) A satisfactory yeast and mould count should be <50/g. A count $>10^2$/g suggests that manufacturing methods are unsatisfactory. The American Butter Institute (1937) recommended that the yeast and mould count should not exceed 10/g. (3) Since the coli-aerogenes content is a direct measure of contamination, the level should be low. To be satisfactory, the colony count on VRB agar should be <10/g, and this has been generally recommended. The presence of 10^2 or more /g indicates serious contamination. As the result of this test is available within 24 hr control measures can be put in hand promptly.

These recommended standards are for freshly manufactured creamery butter examined within 24 hr, since it is obviously desirable that butter should be as free as possible from bacterial contamination when it is packed. It is for this reason that high standards in the various tests are recommended. It has been shown in the present work that these microbial levels can be achieved under modern conditions of manufacture, provided the standard of hygiene in the creamery is good. Further, the testing of butter at an early stage, and the rapid detection of faults, should greatly assist creameries in putting a consistently high quality product on the market.

The three microbial tests recommended are simple and can easily be carried out at creamery laboratories equipped for normal bacteriological control work on milk and milk plant. When used regularly in conjunction with a keeping quality test on butter samples held at 22° they should provide the butter maker with valuable information which will enable him to control and improve the flavour and keeping quality of the butter.

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COLD-TOLERANT FERMENTATIVE GRAM-NEGATIVE ORGANISMS FROM MEAT AND OTHER SOURCES

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SUMMARY: From various chilled meats, twenty-eight strains of coli-aerogenes bacteria and one *Aeromonas* were isolated which grew well at +1·5° and some at −1·5°. The optimum growth temperature for most of these strains was nearer 37° than 30°. Nine strains (including the *Aeromonas*) fermented lactose rapidly, the remainder slowly or not at all. All the strains which fermented lactose rapidly with the production of gas gave positive presumptive coli-aerogenes tests in MacConkey's broth at 30°, but only five were positive at 37°; none was positive at 44°. Because such organisms can attain populations of millions/cm², they could confuse the interpretation of presumptive coli-aerogenes tests made on chilled meat.

Fermentative Gram-negative rods, typified by the coli-aerogenes group, are generally regarded as mesophilic. This term usually implies that their growth will cease at some temperature between 10° and 5°. There are nevertheless a few observations, some of long standing, which demonstrate the ability of such organisms to grow at lower temperatures. Haines (1933) isolated *Proteus* from the slime which developed on meat stored for 1 week at 0–5°. However, although the *Proteus* count on agar plates incubated at 20° was 3×10^9/cm² out of a total population of nearly 3×10^{10}/cm², no *Proteus* were recovered on plates incubated at 4° when the total count was 1×10^{10}/cm². In an investigation on the minimum growth temperature of some bacteria, Haines (1934) found that a strain of *Aerobacter* grew in 36 days at 5·4° but not in 3 months at 0·5°; one strain of *Escherichia* grew in something over 30 days at −2° but two others did not at 5·4°; two strains of *Proteus* grew at 0° (one at −2°) and two strains did not grow. Jensen & Hess (1941) isolated from soured hams strains of *Proteus* and *Serratia* able to grow between 1·1 and 3·3°; but included in the strains identified as *Serratia* was one which failed to ferment glucose. Sulzbacher (1950) isolated from frozen lamb two paracolon organisms able to grow at −6°. Kirsch, Berry, Baldwin & Foster (1952) isolated *Proteus* in considerable numbers from a putrid hamburger which had been stored at 0–2° for approximately 2 weeks. There are several recorded instances of members of the Enterobacteriaceae growing in milk or milk products in the range 3–5° (Thomas, 1958). In most cases, the lower limits of growth of these organisms have not been determined, but Greene & Jezeski (1954) reported a generation time of 37·7 hr at 0° for a strain of *Aerobacter aerogenes* which had an optimum temperature between 30 and 35°. There have been some instances of cold-tolerant coli-aerogenes and related organisms being isolated from cases of fatal or severe reactions following blood transfusion. Pittman (1953) described the isolation of paracolon strains able to grow at 2–5° and McEntegart (1956) found organisms resembling *Klebsiella cloacae* which were able to grow at 4°.

It seems clear that some organisms in this group possess an unusual degree of

tolerance to cold, greater than that accepted for mesophiles, and approaching that regarded as characteristic of psychrophiles. This has been emphasized by the isolation from chilled meats, in the course of several independent investigations in this laboratory, of strains of fermentative, Gram-negative rods which are able to grow well at +1·5° and some at −1·5°. The salient features of these organisms are described in this paper.

Sources of the Organisms

The sources of the strains (29 in all), together with those of other organisms used for comparative purposes, are given in Table 1. The poultry strains were selected from a large number of Gram-negative rods by their ability to ferment glucose (Hugh & Leifson, 1953). In the poultry stored at +3°, the total count rose during storage from about 10^5 to 10^8-10^9/swab, the fermentative strains forming 2–20% of the total

Table 1. *Sources of the organisms studied*

Strain no.	Source
7, 8, 9, 10, 11	Beef: swabbed after one day in butcher's faulty cabinet and 2 days rail journey, followed by 12 days at +1° (1956)
21	Pork: predominant in slime forming at 15° (Kitchell & Ingram, 1956)
44, 46, 49, 89	Whale meat blocks, before and after freezing, and meat plant (1948–9)
55, 57, 90	Fresh whale meat stored in ice (1949)
88	Canned and refrigerated fresh whale meat (1949)
79, 82, 84, 92, 93, 94, 95	Eviscerated poultry stored at +3° (1957, M. J. Thornley)
100, 101, 102, 103, 104, 105, 106, 107	Poultry plant and processed poultry—from plates at +1° (1958, E. M. Barnes)
Aerobacter aerogenes NCIB 8017, 8521	National collection of Industrial Bacteria
Escherichia coli I NCTC 8196 *Citrobacter freundii* I NCTC 8165 *Citrobacter freundii* II NCTC 6071	National Collection of Type Cultures
Aerobacter cloacae 11	Milk — From G. Elis Jones, NAAS, Trumpington, Cambridge
Aerobacter cloacae 12	Plant material — From G. Elis Jones, NAAS, Trumpington, Cambridge
Escherichia coli (44° −ve) 14	Water — From G. Elis Jones, NAAS, Trumpington, Cambridge
Escherichia coli (44° +ve) 16	Water — From G. Elis Jones, NAAS, Trumpington, Cambridge
Aerobacter aerogenes I 26	Milk — From G. Elis Jones, NAAS, Trumpington, Cambridge
Citrobacter freundii I 28	Milk — From G. Elis Jones, NAAS, Trumpington, Cambridge
Klebsiella (Irregular VI) 33	Ropy milk — From G. Elis Jones, NAAS, Trumpington, Cambridge

(Thornley, unpublished results). There is, therefore, every indication that they had multiplied during storage. The remainder of the cold-tolerant strains were fermentative organisms isolated, without their significance being realised at the time, during the course of earlier work and maintained in this laboratory.

Results

Identity of the organisms

All the 29 strains were Gram-negative rods. Six of them were non-motile (44, 46, 49, 101, 104, 107). Of the remainder, all but one (95) had peritrichous flagella. Strain 95 was characterized by having a single polar flagellum and, because of this and the

production of acetylmethylcarbinol, it has been assigned to the genus *Aeromonas*. Eight of the remaining 28 strains fermented lactose rapidly (as did also strain 95), one (88) anaerogenically (Table 2). On the basis of the tests recommended in Report (1958), all 28 strains have been assigned to the coli-aerogenes group as delimited in Report (1956). The allocation of the strains to genera within this group is shown in Table 2, in which anomalous reactions are also listed. It was not possible to assign four strains to genera: the relevant reactions (Report, 1958) of these strains are shown in Table 3.

Table 2. *The classification of* 28 *strains of cold-tolerant coli-aerogenes organisms*

Strain no.	Lactose fermentation	Classification (Report, 1958)	Anomalous reactions
8	Rapid	*Citrobacter*	Malonate +ve
44, 46, 49	Rapid	*Klebsiella*	None
88, 90, 92, 102	Rapid	*Aerobacter*	88, anaerogenic 102, KCN −ve
21, 79, 82, 93, 100, 103, 105, 106	Late or absent	*Aerobacter*	None
7, 9, 10, 11, 55, 57, 84, 94	Late or absent	*Hafnia*	7, MR −ve at 37°, citrate −ve at 20° 9, adonitol +ve, V-P −ve at 20° 10, indol +ve, V-P −ve at 20° 84 & 94, adonitol +ve, MR +ve, V-P weak at 20°
89, 101, 104, 107	Late or absent	Unclassified	See Table 3

Table 3. *The biochemical activities of* 4 *strains which could not readily be assigned to genera according to Report,* 1958

Test	Strain 89	Strains 101, 104, 107
Gas from glucose	−(37° and 20°)	Trace (37°), +(20°)
Lactose	−	Late
Sucrose	+	+
Mannitol	+	+
Dulcitol	−	−
Salicin	−	+
Adonitol	−	−
Inositol	−	+
Indol	−	+
MR	+(37°), −(20°)	+(37°), −(20°)
V-P	+(37°), −(20°)	−(37°), +(20°)
Ammonium citrate	−(37°), +(20° but very slow)	−(20° and 37°)
H_2S	−	−
Urease	−	+
Gelatin	+	−
KCN	−	−
Motility	+	−(20° and 37°)
Phenylalanine deaminase	−	−
Malonate	+	−

Temperature relations

The organisms were able to grow over a considerable temperature range. All grew with acid production in glucose peptone water within 24 hr at 37°. The abilities of the organisms to grow and produce acid in the same medium at +1·5° and −1·5° are shown in Table 4, with the behaviour of some other coli-aerogenes strains for comparison. Ability to grow at −1·5° was less well marked among the lactose-fermenting strains (8, 44, 46, 49, 88, 90, 92, 95 and 102) than among the paracolon types.

Table 4. *The ability of fermentative Gram-negative rods to grow and form acid and gas in glucose peptone water (with Andrade's indicator) at +1·5° and −1·5°*

Strain	Reaction* obtained after incubation at											
	+1·5° for (days):						−1·5° for (days):					
	7	14	21	28	35	42	7	14	21	28	35	42
8	tr A	A	§	§	§	§	—	—	—	—	—	—
44	tr A	AG	§	§	§	§	—	—	—	—	—	—
46	tr A	A	§	§	§	§	—	—	—	—	—	—
49	tr A	A	§	§	§	§	—	—	—	—	—	—
88	A	§	§	§	§	§	—	—	tr A	tr A	A	§
90	AG	§	§	§	§	§	—	tr A	A	§	§	§
92	AG	§	§	§	§	§	—	—	—	—	—	—
95	AG	§	§	§	§	§	—	—	—	—	—	—
102	A	§	§	§	§	§	—	—	—	—	—	—
7	AG	§	§	§	§	§	—	—	tr A	A	§	§
9	AG	§	§	§	§	§	—	tr A	A	§	§	§
10	AG	§	§	§	§	§	—	tr A	A	§	§	§
11	A	§	§	§	§	§	—	—	tr A	tr A	A	§
21	A	AG	§	§	§	§	—	—	tr A	tr A	A	§
55	AG	§	§	§	§	§	—	—	—	tr A	A	§
57	AG	§	§	§	§	§	—	—	—	tr A	A	§
79	AG	§	§	§	§	§	—	—	tr A	tr A	A	§
82	AG	§	§	§	§	§	—	tr A	A	§	§	§
84	AG	§	§	§	§	§	—	tr A	A	§	§	§
89	tr A	A	§	§	§	§	—	—	—	—	—	—
93	AG	§	§	§	§	§	—	tr A	A	§	§	§
94	AG	§	§	§	§	§	—	tr A	A	§	§	§
100	A	§	§	§	§	§	—	—	tr A	A	§	§
101	A	§	§	§	§	§	—	A	§	§	§	§
103	A	§	§	§	§	§	—	tr A	A	§	§	§
104	A	§	§	§	§	§	—	tr A	A	§	§	§
105	A	§	§	§	§	§	—	tr A	A	§	§	§
106	A	§	§	§	§	§	—	—	—	—	tr A	A
107	A	§	§	§	§	§	—	tr A	A	§	§	§
A. aerogenes NCIB 8017	—	—	tr A	tr A	A	§	—	—	—	—	—	—
A. aerogenes NCIB 8521	—	—	tr A	tr A	A	§	—	—	—	—	—	—
E. coli I NCTC 8196	—	—	—	—	—	—	—	—	—	—	—	—
Cit. freundii I NCTC 8165	—	—	—	—	—	—	—	—	—	—	—	—
Cit. freundii II NCTC 6071	—	—	—	—	—	—	—	—	—	—	—	—
A. cloacae 11	—	tr A	AG	—	—	—	—	—	—	—	—	—
A. cloacae 12	AG	§	§	§	§	§	—	—	—	tr A	A	§
E. coli (44° −ve) 14	—	—	—	—	—	—	—	—	—	—	—	—
E. coli (44° +ve) 16	—	—	—	—	—	—	—	—	—	—	—	—
A. aerogenes I 26	AG	§	§	§	§	§	—	—	—	—	—	—
Cit. freundii I 28	tr A	A	§	§	§	§	—	—	—	—	—	—
Klebsiella (Irreg. VI) 33	—	—	—	—	—	—	—	—	—	—	—	—

* —, no growth; A, acid; G, gas; tr, trace., § no further change;

The optimum temperatures of the organisms were determined by timing the first appearance of visible turbidity in tubes of glucose broth incubated at 20°, 30° and 37°. The results are in Table 5. In spite of the cold-tolerance exhibited by these

organisms, the majority grew faster at 37° than at 30°. Comparison of Tables 4 and 5 shows that there is no apparent correlation between optimum temperature and degree of cold-tolerance.

Table 5. *The optimum growth temperatures of 29 strains of cold-tolerant fermentative Gram-negative organisms*

Optimum growth temperature (°C)	Strains
ca. 37	7, 8, 9, 10, 11, 21, 44, 46, 49, 55, 57, 84, 88, 89, 90, 92, 94, 95, 100, 101, 104, 106, 107
ca. 30	79, 82, 93, 102, 103, 105
ca. 20	None

Presumptive coli-aerogenes test

Because most of these organisms had an optimum temperature of 37°, it was of interest to find out how the lactose-fermenting strains would behave in the presumptive coli-aerogenes test. Accordingly, the nine such strains were inoculated into MacConkey's broth and brilliant green bile broth and incubated at 37° and 30°. Two strains of *Aerobacter aerogenes* were included for comparison. The results are shown in Table 6. None of the strains grew in MacConkey's broth in 24 hr at 44°.

Table 6. *The behaviour of lactose-fermenting cold-tolerant Gram-negative rods in the presumptive coli-aerogenes test*

Strain	Reaction* after 48 hr in			
	MacConkey's broth at		Brilliant green bile broth at	
	37°	30°	37°	30°
9	AG	AG	G	G
44	AG	AG	G	G
46	AG	AG	G	G
49	AG	AG	G	G
88	A	A	g	g
90	A	AG	n.g.	G
92	AG	AG	n.g.	G
95	A	AG	g	g
102	n.g.	AG	n.g.	G
A. aerogenes NCIB 8017	AG	AG	G	G
A. aerogenes NCIB 8521	AG	AG	G	G

* A, acid; G, gas; g, growth; n.g., no growth.

Discussion

The fact that the optimum temperature of most of the strains was 37° indicates that these organisms are not typical psychrophiles but should more accurately be described as cold-tolerant mesophiles. The paracolon organisms isolated by Sulzbacher (1950) were unable to grow at 37°. It is noteworthy that, although most of them

fermented lactose slowly or not at all (i.e. are of paracolon type), they nevertheless include several typical coli-aerogenes organisms.

Interest attaches to the isolation of fermentative Gram-negative rods (and particularly of members of the coli-aerogenes group) able to grow at low temperatures, from chilled meats under conditions where there was a strong presumption that growth had taken place. The chief interest is in relation to the orthodox presumptive coli-aerogenes test as an indicator of faecal pollution. Several of the organisms appear to be true coli-aerogenes bacteria, though some are peculiar in that they give typical reactions in presumptive tests at 30° but not at 37°. This phenomenon is well known in other fields (Thomas, 1955). The use of incubation at 30° rather than 37° is likely to yield more positives, but it seems that the significance of presumptive coli-aerogenes bacteria recovered at the lower temperature may be questionable.

However the presumptive test be carried out, the fact that several cold-tolerant organisms are capable of giving a positive reaction suggests that, were the test to be applied to stored chilled meat, gross faecal contamination might be suspected. To infer gross contamination would clearly be unsound, since the organisms might well have multiplied greatly during storage. Nevertheless, it is conceivable that these organisms might be present because of faecal contamination. The validity of this presumption, however, would clearly rest on the occurrence of similar organisms in the gut of humans or other relevant animals; but it seems, in fact, that they may not be common there. Search in the gut of chickens has failed to reveal any (E. M. Barnes, unpublished observations). Their true origin has still to be discovered.

The absence from chilled meat of 44° positive types would be of no significance. After storage at low temperature, these would be expected to form an insignificant fraction of the total population, due either to death of such cold-intolerant strains, or to overwhelming growth of the cold-tolerant strains, or both. The presence of 44° positive strains would, on the other hand, retain its significance. It is common practice to confirm presumptive coli-aerogenes reactions by incubation of cultures in MacConkey's broth at 44°. None of the present collection of cold-tolerant strains passed this test. Thus, their existence does not call into question the validity of indications based on confirmatory tests at 44°; indeed, it rather emphasizes the desirability of such confirmation, especially in making sanitary evaluations of chilled meats.

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SOME OBSERVATIONS ON BACTERICIDAL PAINT FILMS

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SUMMARY: A method is described for testing the self-sterilizing activity of surfaces coated with paints containing bactericidal agents. The most active coating consisted of 10% (w/w) Cetavlon dissolved directly in urea-formaldehyde:alkyd resin. The recovery of viable organisms from such surfaces infected with *Streptococcus faecalis*, *Staphylococcus aureus*, *Aerobacter aerogenes* and *Chromobacterium prodigiosum* was less than 10% of that from painted control surfaces after exposure for 1 hr at atmospheric relative humidity of 70% and temperature of 20°. *Bacillus subtilis* spores were not affected until they became heat-sensitive. The self-sterilizing activity was markedly diminished at 40% r.h. and the inclusion of blood in the deposited bacterial particle was then completely protective. Repeated washing in water with gentle rubbing quickly inactivated Cetavlon paint films.

Self-sterilizing surfaces would assist in controlling the bacterial flora in conjunction with air disinfection measures. The development of surfaces capable of such activity has received considerable attention, but little success has been found in their practical use. It was therefore considered pertinent to study the mechanisms involved in self-sterilization and to assess the practical value of bactericidal paints. The earlier methods of testing paints, employing bacteria in a wet form, altered the composition of the paint film and often enhanced the killing effect. The tests on the inhibitory activity of surfaces did not always indicate their bactericidal activity. Epstein & Snell (1941) reviewed these test methods and discussed the results obtained by various workers. In recent years the development of methods employing bacterial mists has enabled surfaces to be infected in a more natural manner. Suspensions of bacteria are atomized as a fine spray, which is then allowed to settle on the test surfaces. The particles in the aerosol rapidly achieve equilibrium with the ambient humidity and the effect of atmospheric relative humidity on the killing rate can thus be studied. Annear (1951) used this method for infecting glass slides with streptococci: subsequently the organisms were cultivated by placing the slides on an agar surface. Hoffman, Yeager & Kaye (1955) held the infected surfaces in constant humidity tubes containing small quantities of saturated solutions of inorganic salts, and the bacteria were enumerated by shaking the test slides in a neutralizing fluid and plating this on solid media. The role of relative humidity in the self-sterilization of surfaces was studied by Dunklin & Lester (1951) and by Hoffman (1951).

The present paper is not concerned with the chemical or physical aspects of paint formulation but with the antibacterial activities of surfaces coated with paints to which bactericidal substances have been added. An attempt has been made to apply a quantitative method for comparing the activities of various formulations. The method is similar to that of Hoffman, Yeager & Kaye (1955) but the control of

humidity has been simplified so as to enable a large number of surfaces to be tested at one time.

Materials and Methods

Paint media. The basal paint media consisted of 50:50 mixtures of urea-formaldehyde:alkyd resins and a number of standardized bases obtained from the Ministry of Supply Chemical Inspectorate, comprising epoxide and alkyd resins, gloss and flat finishes and a polyvinyl acetate emulsion.

Bactericides. These comprised: Cetavlon (Cetrimide, B.P., cetyl-trimethylammonium bromide, Imperial Chemical Industries, Ltd.); lauryl-pyridinium chloride; cetyl-pyridinium chloride; cetyl-pyridinium bromide; cetyl-pyridinium iodide; 'Arquad' 2C and 2HT (Armour & Co., Ltd.); 'Tego' MHG, 51 and 103S (Hough, Hoseason & Co., Ltd.); *o*-hydroxydiphenol; toluene-*p*-sulphonamide; pentachlorophenol; phenylmercuric acetate; tetramethylthiuram disulphide; paraformaldehyde; hexachlorophene; hexamine; 'Hibitane'; tri-*n*-butylin oxide (Pure Chemicals, Ltd.), laurate, naphthenate and resinate; and 'Dentolite' self-sterilizing paint.

Preparation of coatings. Bactericidal substances were dissolved directly in the paint media, usually in 10% (w/w) concentration. The effect of these additions on the physical properties of the paint was not considered, but only coatings of good finished appearance were used. The test paints and control coatings of basal paint media were applied with a camel hair brush to one side of thin white cardboard. After the appropriate drying period in air, at 100° or at 170°, the cards were cut into 1 in. squares and stored at room temperature in modified slide holders for 14 days before testing.

Exposure chamber. This was a rectangular wooden box of 14 ft^3 capacity, painted inside with a white chemical-resistant paint. Observation windows were fitted in the top and three sides of the chamber. Internal fittings included an ultraviolet light sterilizing tube, a small mixing fan, wet- and dry-bulb thermometers and connections to compressed air and steam. A metal rod for remote handling was passed through a rubber diaphragm in one side of the chamber and was used for exposing the paints by removing the sliding lid covering a tray containing the test surfaces. Negative pressure could be maintained in the chamber with a vacuum pump which extracted air through a bacterial filter.

Dispersal of bacteria. Suspensions were made of *Streptococcus faecalis*, *Staphylococcus aureus*, *Aerobacter aerogenes*, *Chromobacterium prodigiosum* and *Bacillus subtilis* spores, by washing off growth on agar with water or other fluid. In the case of *B. subtilis* the suspension was heated at 60° for 1 hr. Most of the work was done with suspensions in 1% (w/v) glycerol in a phosphate buffer consisting of: KH_2PO_4, 4·5 g/l.. $(NH_4)_2SO_4$, 0·5 g/l. and NH_4Cl, 0·5 g/l., adjusted to pH 7·6 by the addition of NaOH, *c.* 3 ml of a 40% (w/v) solution/l. The glycerol delayed drying of the bacterial particles and so improved the viability of the organisms on the control surfaces. The suspensions were diluted and pooled to give *c.* 2×10^9 of each vegetative organism/ml and *c.* 2×10^8 of *B. subtilis* spores/ml and were sprayed from an atomizer of the scent-spray type moderately baffled to produce from aqueous suspensions particles 10–15 μ in diameter after evaporation.

Test procedure. Three squares of each bactericidal paint and three control squares of each basal paint were held with a small piece of plasticine in randomized positions on a metal tray. A total of 30 squares was used for each test. The tray was covered with a loose-fitting lid and placed centrally in the exposure chamber. Then the atomizer containing the pooled bacterial suspension was placed inside the chamber and the mixing fan turned on. Wet- and dry-bulb thermometers were positioned as near to the fan as possible and temperature readings made through a window in the chamber. The vacuum pump was then started, and at a controlled negative pressure of 2 in. of water the humidity conditions were adjusted by admitting steam or dry air. After the conditions inside the chamber had been maintained for several minutes, bacteria were dispersed from the atomizer for 5 min. This caused a temporary rise of less than 10% r.h. for about 10 min only. The mixing fan was stopped at the end of the spraying period and, after a 1 min wait to allow gross particles to deposit, the lid covering the paint surfaces was removed for 10 min to allow bacteria to settle on the paints. After replacing the lid the interior of the chamber was exposed to ultraviolet radiation for 10 min and the vacuum pump switched off. The test surfaces were left in the chamber at atmospheric pressure for 1 hr, the humidity being adjusted if necessary. The temperature in the chamber remained between 19° and 20°, slightly above the controlled temperature of the laboratory.

Sampling and counting technique. Media selective for the growth of each organism in the pooled suspension were needed for the assessment of viable organisms from the test surfaces. It was found by experiment that the following selective substances, added to a tryptic digest of beef agar, allowed the quantitative growth of the various species: for *Chr. prodigiosum*, dibromopropamidine (May & Baker, Ltd.) at 12·5 μg/ml; for *Strep. faecalis* and *Staph. aureus*, sodium azide at 100 μg/ml; for *A. aerogenes*, hydroxylamine acetic acid at 170 μg/ml and phenol at 800 μg/ml. On these media the colonies of the different species were identical in appearance and number with those grown on the basal nutrient agar. Incubation was at 37° for 18 hr. A highly pigmented strain of *Staph. aureus* was chosen for this work to enable rapid differentiation of the colonies from those of *Strep. faecalis*, which also grew on the azide medium.

Two methods of sampling the test surfaces were compared. The first method consisted of rubbing the coated squares with alginate wool swabs moistened with nutrient broth. These swabs were dissolved in sodium citrate solution, which was then plated on the selective media. The numbers of organisms recovered by this method were very low, and they sometimes varied widely from identical surfaces. In the method finally adopted, each painted square was immersed in 10 ml of a sampling fluid and vigorously shaken for a few moments to remove the deposited organisms. A small quantity of the bactericide was washed out of the test paints by this method and inhibition of the more sensitive species sometimes occurred, but by adding known concentrations of bactericide to suspensions of the test organisms containing various neutralizing substances, a sampling fluid which would neutralize any possible inhibitory effect was devised. This contained the yolk of 1 egg emulsified in 500 ml of tryptic digest broth with 0·1% (v/v) of Tween 20 added. It neutralized twice the amount of the most active of the bactericides used that could be washed out of the painted surfaces in the time allowed. The Tween 20 was used to improve the bacterial

sampling efficiency and had no effect on the growth of the organisms.

Immediately after shaking the test squares, 5 ml portions of the sampling fluids were removed. In the case of fluids from control surfaces, a part of the sample was diluted 1:50 in egg yolk broth. The undiluted fluids from the bactericidal paints and the diluted fluids from the control paints were then plated in triplicate on each sclective medium by the Miles & Misra (1938) method, using inocula of 6 drops/plate from a 36 drops/ml standardized pipette. A total of 0·5 ml of fluid, therefore, was plated from each painted square. When *B. subtilis* spores were included, the fluids were also heated at 50° for 45 min and plated on nutrient agar. The plates were incubated for 18 hr at 37°, when the colonies of each species were counted. On several occasions the plates were re-counted after a further 24 hr incubation, but no increase was found. The numbers of colonies from the 3 identical squares of each bactericidal paint (i.e. the numbers from 9 plates) were expressed as percentages of those, previously multiplied by 50, obtained from the diluted sampling fluids of the 3 appropriate control squares. Since the control and test surfaces were exposed at the same time these percentages represent the effect due to the bactericide alone.

Results

Reproducibility of the results

The reproducibility of the results is illustrated in Table 1. The numbers of organisms recovered from 30 identical squares of a control paint were closely similar, and it is therefore considered that the method was adequate for detecting gross differences in the activities of bactericidal paints. The atmospheric humidity in the exposure chamber was not adjusted during these recovery experiments and varied from 45% to 60% r.h. Changes in the r.h. affected the numbers of organisms recovered, but the counts from identical surfaces in any one experiment were always closely similar.

Table 1. *The recovery of organisms from inoculated control surfaces (painted squares) of* 50:50 *urea-formaldehyde:alkyd resin*

(Atmospheric r.h., 45%; spray fluid, 1% glycerol in buffer, see text)

Square no.	Organisms recovered (10^5/square)					Square no.	Organisms recovered (10^5/square)				
	f	p	a	au	s		f	p	a	au	s
1	12·27	3·60	1·20	1·05	6·24	16	10·31	3·93	1·16	0·81	6·48
2	10·06	4·17	1·44	1·11	6·15	17	12·24	3·81	0·86	1·02	6·16
3	12·22	3·63	1·20	0·55	6·31	18	11·21	3·75	1·09	0·78	6·90
4	11·25	3·90	1·05	0·69	6·19	19	14·03	3·90	1·01	1·15	6·16
5	14·20	3·84	0·99	0·54	6·12	20	10·90	3·48	1·31	0·56	6·37
6	13·97	3·45	1·20	1·14	6·25	21	10·76	3·39	0·77	0·75	6·31
7	13·34	3·72	1·23	0·93	6·24	22	10·85	3·57	0·93	0·87	6·52
8	11·58	4·08	0·61	0·67	6·15	23	11·41	3·51	1·18	1·13	6·48
9	9·74	3·18	0·51	0·36	6·20	24	10·36	3·36	0·88	0·97	6·27
10	9·53	3·81	0·66	0·54	6·48	25	11·13	3·78	1·14	0·73	6·19
11	10·50	3·48	0·45	0·75	6·33	26	9·22	3·21	1·17	0·88	6·43
12	10·62	3·72	1·20	0·84	6·31	27	11·07	3·33	0·93	1·01	6·28
13	10·26	3·93	0·65	0·96	6·52	28	9·01	3·42	0·88	0·74	6·12
14	10·04	3·66	1·22	1·11	6·31	29	10·52	3·21	0·69	0·65	6·24
15	10·37	3·42	0·97	0·82	6·35	30	9·95	3·63	1·03	0·90	6·25

* f, *Strep. faecalis*; p, *Chr. prodigiosum*; a, *A. aerogenes*; au, *Staph. aureus*; s, *B. subtilis* spores.

The recovery also varied with the type of control paint and with changes in the constituents of the spray fluid. It was therefore essential to include in every experiment the appropriate control surfaces for each test paint so that the variable control recovery and control death of the deposited organisms could be separated from the effect of the bactericide. Examples of the recovery of viable organisms from various control surfaces are given in Table 2.

Table 2. *The effect of the spray fluid on the recovery of* Strep. faecalis *from various control surfaces*

Type of surface*	Relative humidity (%)	Mean recovery (10^5/square) with spray fluid†			
		A	B	C	D
50:50 UF:alkyd	70	9·32	4·67	6·38	6·87
	40	14·43	8·14	8·03	14·21
PVA emulsion	70	5·31	0·73	3·32	1·63
	40	10·81	5·31	5·57	10·50
Alkyd gloss	70	11·35	5·69	5·16	7·06
	40	14·40	5·13	8·69	1·22
Hospital enamel	70	8·64	4·78	4·24	7·94
	40	13·46	6·73	6·95	12·37

* UF, urea-formaldehyde; PVA, polyvinyl acetate.

† A, 1% glycerol in buffer; B, distilled water; C, 20% blood in physiological saline; D, saliva.

The effect of bactericidal substances on the recovery of organisms

The results of tests on a number of quaternary ammonium compounds and related substances incorporated in urea-formaldehyde:alkyd resin, are shown in Table 3. Cetavlon was the most active, the recovery being only *c.* 1% of that from control squares after 1 hr exposure at 70% atmospheric relative humidity. The activities of

Table 3. *The effect of various quarternary ammonium bactericides in paint on the recovery of organisms from painted squares*

(Atmospheric r.h., 70%; spray fluid, 1% glycerol in buffer)

Bactericide*	No. of replicate tests	Mean recoveries (% of control)			
		f†	p	a	au
'Cetavlon'	15	0·6	1·0	0·9	0·2
Lauryl-pyridinium chloride	4	39	12	8	5
Cetyl-pyridinium chloride	4	53	29	18	2
Cetyl-pyridinium bromide	3	65	51	43	21
Cetyl-pyridinium iodide	7	41	27	10	—‡
'Arquad' 2C-75	3	48	76	47	25
'Arquad' 2HT-75	3	48	67	14	23
'Tego' MHG	2	21	6	2	8
'Tego' 51	2	20	3	2	10
'Tego' 103 S	2	16	3	2	4

* 10% (w/w) of each substance was incorporated in 50:50 urea-formaldehyde: alkyd resin.

† f, *Strep. faecalis*; p, *Chr. prodigiosum*; a, *A. aerogenes*; au, *Staph. aureus*.

‡ not included.

various other substances are given in Table 4. They were low in comparison with those of the quaternaries.

Table 4. *The effect of bactericides, other than quaternaries, in paint on the recovery of organisms from painted squares*

(Atmospheric r.h., 70%; spray fluid, 1% glycerol in buffer)

Bactericide*	No. of replicate tests	Mean recoveries (% of control)			
		f†	p	a	au
o-hydroxydiphenol	3	69	78	72	—‡
toluene-*p*-sulphonamide	2	73	81	63	—
pentachlorophenol, 5%	2	100	78	37	—
phenylmercuric acetate	4	52	57	25	—
tetramethylthiuram disulphide, 5%	2	54	44	35	—
paraformaldehyde	4	33	29	10	6
hexachlorophene, 20%	2	100	100	100	100
hexamine, 5%	2	75	82	39	51
'Hibitane', 5%	4	45	96	51	—
tri-*n*-butylin oxide	2	18	32	12	11
tri-*n*-butylin laurate	2	46	27	12	8
tri-*n*-butylin naphthenate	2	30	19	8	5
tri-*n*-butylin resinate	2	23	18	8	5
'Dentolite' self-sterilizing paint	2	75	60	86	—

* 10% (w/w) of each substance was incorporated in 50:50 urea-formaldehyde:alkyd resin, unless otherwise stated.

† f, *Strep. faecalis;* p, *Chr. prodigiosum;* a, *A. aerogenes;* au, *Staph. aureus.*

‡ not included.

The effect of the basal paint medium on the activity of Cetavlon paint

Differences in bactericidal activity were found when 10% Cetavlon was incorporated in various basal paint media (Table 5). It is possible that physical factors involved in the formation of the final surface influenced the activity. The extremely hard surface of the epoxide resins, whether heat or air dried, were non-bactericidal, while the relatively softer surfaces were very active. The most active coating was from

Table 5. *The effect of the basal paint medium on the recovery of organisms from Cetavlon paints*

(Atmospheric r.h., 70%; spray fluid, 1% glycerol in buffer)

Basal paint*	Nature of surface	No. of replicate tests	Mean recovery (% of control)			
			f†	p	a	au
Stove dried epoxide	Very hard, glossy	2	100	100	100	100
Cold cured epoxide	Very hard, glossy	2	100	100	100	100
Stove dried alkyd	Hard glossy	4	0·6	2·0	0·6	0·5
Flat wall finish	Soft, matt	2	3·7	2·7	5·0	3·7
Polyvinyl emulsion	Soft, matt	4	5·7	6·8	4·0	8·7
Alkyd gloss	Hard, glossy	4	4·1	—‡	—	3·5
Hospital enamel	Semi-hard, glossy	4	2·2	3·2	2·1	1·5
Hospital enamel B	Semi-hard, glossy	2	3·0	2·5	1·8	2·0
50:50 UF:alkyd resin	Semi-hard, glossy	4	0·5	1·1	0·4	0·2

* 10% (w/w) Cetavlon was incorporated in each basal paint. ‡ —, not included.

† f, *Strep. faecalis;* p, *Chr. prodigiosum;* a, *A. aerogenes;* au, *Staph. aureus.*

50:50 urea-formaldehyde:alkyd resin, which dried with a relatively soft glossy surface. Among the other surfaces the gloss finishes were more active than the matt, of which polyvinyl acetate emulsion had the least action. During these experiments it was noted that the alkyd gloss paint was itself somewhat bactericidal for *Chr. prodigiosum* and *A. aerogenes*. Numbers of these organisms sampled were only *c.* 50% of those obtained from other glossy surfaces such as the urea-formaldehyde:alkyd resin. The other test organisms were not affected.

The effects of the nature of the spray fluid and of atmospheric relative humidity

Under natural conditions of contamination, bacteria would often be deposited on surfaces from suspensions containing organic material and would tend to be protected. Experiments were therefore carried out on Cetavlon coatings, with the test organisms sprayed from fluids containing blood, peptone, or saliva. Suspensions of the organisms were prepared by washing off growth on agar with the test fluids. Saliva was first treated by heating for 1 hr at 60° and filtering through glass wool. When blood was present it was not possible to prepare a stable suspension of *Staph. aureus* because the cells clumped, and this species was omitted. It will be seen from the results, summarized in Table 6, that at 70% atmospheric relative humidity there was no marked effect on activity. Blood appeared to be the most protective substance, particularly on the polyvinyl acetate emulsion paint.

Table 6. *The effect of the spray fluid on the recovery of organisms from paints containing* 10% *Cetavlon at r.h. values of* 70% *and* 40%

Basal paint	Mean percentage recoveries* of different organisms dispersed in																			
	1% glycerol in buffer				Water				20% blood in saline			0·5% peptone water				Saliva			
	f†	p	a	au	f	p	a	au	f	p	a	f	p	a	au	f	p	a	au
Atmospheric r.h., 70%																			
50:50 UF:alkyd	0·5	1·1	0·4	0·2	0·5	0·5	0·1	0·2	0·7	0·4	0·1	0·4	1·2	0·1	0·5	0·6	0·5	0·1	0·1
Stove dried alkyd	0·6	2·0	0·6	0·5	1·2	2·0	1·1	0·7	2·2	2·5	3·8	1·0	0·6	0·3	0·9	1·0	1·5	0·5	1·3
PVA emulsion	5·7	6·7	4·0	8·7	5·7	5·9	2·1	4·5	10·8	13·6	11·6	9·7	2·5	2·7	11·9	7·0	8·6	1·6	9·0
Alkyd gloss	4·1	—‡	—	3·5	2·6	—	—	1·1	7·8	—	—	3·6	—	—	2·5	7·9	—	—	3·5
Hospital enamel	2·2	3·2	2·1	1·5	1·2	3·6	0·9	3·3	3·9	4·0	3·8	1·4	0·7	0·3	1·5	2·1	3·4	2·3	1·8
Atmospheric r.h., 40%																			
50:50 UF:alkyd	13	9	10	1	57	65	65	36	71	100	96	—	—	—	—	64	77	70	45
Stove dried alkyd	10	14	24	10	79	97	85	47	84	100	100	—	—	—	—	57	77	61	44
PVA emulsion	3	8	8	3	26	58	59	47	54	100	100	—	—	—	—	40	74	53	38
Alkyd gloss	30	32	36	31	77	100	100	66	94	100	100	—	—	—	—	93	100	100	100
Hospital enamel	22	38	36	35	80	90	77	90	100	100	100	—	—	—	—	84	100	89	82

* These were from four replicate trials with each basal paint medium.

† f, *Strep. faecalis;* p, *Chr. prodigiosum;* a, *A. aerogenes;* au, *Staph. aureus* —, not included.

Low atmospheric humidity markedly reduced the bactericidal action of Cetavlon coatings. In experiments conducted at 40% r.h. the recovery from the paints inoculated from aqueous suspensions of bacteria was over 60%, while bacteria sprayed from fluids containing blood or saliva were almost completely protected (Table 6). There was some bactericidal action with buffer-glycerol suspensions due to the higher humidity of the bacterial particles, but the killing rate was lower than at 70% r.h. Humidity readings were not taken at the surface of the paints and it is probable

that the slightly greater activity shown by the emulsion paint at 40% r.h. was due to the greater time required for equilibrium to be reached with a lowered atmospheric humidity by this matt surface than by the gloss paints. The control alkyd paint had no bactericidal action at 40% r.h. on the two species of organisms previously affected in tests at the higher humidity.

The action of bactericidal paints on B. subtilis *spores*

In experiments with *B. subtilis* spores included in the pooled suspension, where the sampling fluids were heated before being plated, there was a marked reduction in the numbers of spores recovered from bactericidal paints. The tests were then repeated, but with the other test organisms omitted. Glycerol-buffer was used as the spray fluid and the sampling fluids were plated on nutrient agar before and after heating at 50° for 45 min. It was found that heating the fluids from control surfaces halved the numbers of colonies, suggesting that during the 1 hr holding period about 50% of the spores had become heat sensitive. The unheated fluids from Cetavlon paints also produced half the numbers of colonies obtained from the unheated controls, indicating that the bactericide had affected only those altered spores. When the Cetavlon samples were heated, however, they gave only *c.* 10% of the number from the heated controls. This suggests that spores exposed to Cetavlon are sensitive to quite moderate heating. When the holding period was increased to 4 hr, or when the spores were sprayed from nutrient broth in high r.h., the percentage of spores that became heat sensitive was increased. Purely vegetative *B. subtilis* organisms were killed on bactericidal surfaces as rapidly as the other test organisms.

The stability of the bactericidal action of Cetavlon paints

The effects of storage in open containers and of washing the surface of the paints was tested at intervals of 6 months. There was no loss in activity after 2 years storage. Washing in running water and rubbing gently with cotton wool had little effect on activity on the first occasion, but when this was repeated there was a 50% reduction in activity. After 4 washings the paint films were relatively inactive.

Discussion

Cetavlon, which is highly soluble and stable, was the most active bactericide tested. When incorporated in most of the basal paint media it dispersed readily and the dried paints had no visible film on the surface. Epoxide resin was an unsuitable vehicle, possibly because the bactericide was locked beneath the extremely hard surface.

In natural conditions of contamination, bacteria are deposited on surfaces as particles containing different numbers of organisms in association with variable amounts of organic material, or with dust particles. The natural death rate of organisms from such factors as radiation, humidity and temperature would be influenced by the size of the particle, the time of exposure and the species of organism. The death rate on bactericidal surfaces would in addition be limited by the activity

and availability of the bactericide at the surface, and its solubility and diffusion into the bacteria-carrying particle. The concentration of bactericide in the coating of the particle would be governed by the presence of moisture at its point of contact with the paint surface. Organic material in this coating would affect the speed of diffusion of the bactericide. The work described above has illustrated the action of some of these factors.

Only a proportion of the bacteria deposited on the test paints was removed in sampling, but it is likely that the number removed would exceed that which might be displaced from naturally infected surfaces by such normal means as air currents, mechanical rubbing, etc. The test organisms removed from the bactericidal paints, but which failed to grow on the agar media, were assumed to have been killed on the paint, since there was no inhibition of growth from carry-over of bactericide into the inocula and no inhibition on the selective media.

Bactericidal paint films would appear to have only a limited use. The organisms in the larger bacteria-carrying particles sedimenting on them, particularly those embedded in dust or dry organic material, would be killed only slowly in high humidities and not at all in low humidities. Surfaces which became coated with dry particles of dust, soot, etc., would be inactivated against bacteria subsequently deposited. Washing the surface to remove such a coating would diminish the activity of the paint film, the extent of this depending on the solubility of the bactericide used in the paint formulation, and substances of low solubility would need to be extremely active to attain self-sterilization in conditions other than those of very high humidity. The only conditions in which Cetavlon paint films would have any practical value would be dust-free situations where the r.h. was above 60%.

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THE SANITATION OF FISH BOXES. I. THE QUANTITATIVE AND QUALITATIVE BACTERIOLOGY OF COMMERCIAL WOODEN FISH BOXES

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SUMMARY: The cleaning efficiency of hosing wooden market fish boxes with cold water, the usual commercial practice, was very low, even when high pressure jets were used. After washing, the internal surfaces could still carry up to 20×10^6 bacteria/cm^2. Even prolonged steaming failed to sterilize the surfaces. Coryneform organisms accounted for over 50% of the total bacterial flora, although these have usually been reported as present in only low proportions on both fresh and spoiling fish. The major subsidiary groups were species of *Achromobacter* and *Pseudomonas*, constituting about 18% and 14% of the population respectively. A few micrococci and flavobacteria were also present.

The conditions of counting, i.e. whether roll tubes or Petri dishes were used, whether incubation was at 0°, 20° or 37°, and whether the media were based on sea or tap water, could markedly influence both the magnitude of the count and the proportions of the different types of bacteria which could be isolated.

Many studies have been made of the bacterial flora of various species of marine fish and of the quantitative and qualitative changes which take place during storage in ice, i.e. during spoilage (Shewan, 1949), but comparatively little attention has been paid to the bacterial floras of the environments of fish after it has been caught, with the exception of the ice in which fish is stowed on the trawler. This may be heavily contaminated during handling and storage from dirty holds or previous ice supplies (Castell & Triggs, 1953; Report, 1955; Georgala, 1957*a*).

Wood (1939) determined the incidence of various types of bacteria in certain environments, e.g. market air, various surfaces with which fish might come into contact and the fresh water used in washing the fish, and found marked qualitative differences between the floras of different environments. Other investigations have shown considerable levels of contamination of wood chips from the holds of sardine boats and scrapings from canned-sardine packing tables (Highlands & Williams, 1944), brining tanks in filleting plants (Hartshorne, 1947), wash water on the Manila market (Clague & Almario, 1950) and a variety of surfaces in fish processing plants in Canada (Castell, 1954), where marked re-infection of the fish was occurring after filleting.

Despite the fact that fish containers are generally the first source of incidental contamination on land, no attention has been paid to their bacteriology, and one purpose of the present paper is to record the results of an investigation, quantitative and qualitative, of the bacterial flora of one type of wooden fish box, made as part of a general survey of the sanitation of the distributive chain of fish on land.

The two factors of major importance in making colony counts of bacteria are the nutrient medium used and the temperature of incubation, and these factors are also important when it is desired to isolate organisms from mixed populations for study

in pure culture. From the results of work with marine bacteria (ZoBell, 1946) and bacteria from fish (Elliott, 1948; Liston, 1956), it can be said that maximum bacterial counts on samples of fish or on material heavily contaminated with fish bacteria generally occur with a nutrient medium based on sea water and with incubation at 20° or below. No data, however, appear to be available to show the effect of the medium or the temperature of incubation upon the magnitude of the bacterial count of samples taken from the environments of fish on land, where marine and terrestrial bacteria might be expected to exist together, nor which bacterial groups, if any, are selected by particular counting conditions. Accordingly a major object of the work here reported was to determine the effect of several counting conditions on the magnitude of the count obtained from samples taken from commercial wooden fish boxes, and on the types of bacteria isolated.

Roll tubes are becoming increasingly popular for making bacterial colony counts, being easy to use and economical with media; but since in some cases their use may lead to counts differing in magnitude from Petri dish counts on similar samples (Snyder, 1947; Clegg, Thomas & Cox, 1951), roll tube counts were also included in the series, using the Astell apparatus.

Materials and Methods

Fish boxes. The boxes used in the present work were the wooden boxes, of eight stones capacity, in which fish are placed from the trawlers. They are not used at all fishing ports, having been superseded in some cases by aluminium boxes or tubs, but they are used in Aberdeen, the third largest fishing port, where the work described here was carried out, and also in some of the smaller ports. After having been filled the boxes generally lie for several hours on the quayside until the fish are sold, when they are taken by lorry to the fish houses, where the fish is processed.

The boxes used in the tests described here were taken from commercial fish houses soon after being emptied and were sampled, treated, etc., in the laboratory.

Treatment of boxes. The boxes were washed by cold mains water with a low pressure jet, obtained from a hose pipe fitted directly to the mains supply. This was the method of washing used in the Aberdeen Box Pool. One set of boxes, however, was washed with a high pressure jet used by a commercial fish house, again using cold mains water. After washing, the boxes were steamed. For this they were inverted over a steam jet for 1 hr. In some cases, they were then kept for 48 hr in a cool place prior to a final sampling. This period of standing is normal for cleaned boxes in commercial practice.

Sampling the boxes. Two methods were used. In the swabbing method, a marked area of the internal surface, approximately $4{\cdot}8 \times 7{\cdot}8$ cm, was swabbed with alginate wool which was then dissolved in 90 ml of normal strength Ringer's solution plus 1 ml of 10% sodium hexametaphosphate solution. This was mechanically shaken for 10 min and added to 9 ml of Ringer's solution. From this 10^{-2} dilution, further decimal dilutions were made. Ringer's solution was used as the diluent as it compares favourably with aged sea water (Report, 1953). In the scraping method, a similar marked area was thoroughly scraped with a razor blade to which the removable surface material adhered. This material was then wiped off with an alginate swab,

which was then treated as in the swabbing method. Scraping, in contrast to swabbing, removed the surface material down to the hard wood base. In some cases the surface was too dry to allow material to be obtained by scraping alone and in these cases the area was moistened with an alginate swab soaked in sterile tap water before scraping. This swab was dissolved along with that used for cleaning the razor blade. The boxes were sampled in two positions: along the centre board at the bottom and along the centre board at one of the short sides.

Counting conditions. Two media were used. One was an ox- or horse-heart digest tap water agar (OHD) containing tap water extract of minced heart, 1000 ml; NaCl, 5 g; peptone (Oxoid), 10 g and agar, 15 g. The pH was adjusted to 7·6. The other was a modified ZoBell (1946) sea water based agar (ZSW) consisting of Lab-Lemco, 1 g; peptone (Oxoid), 1 g; glucose, 1 g; KH_2PO_4, 0·05 g; aged sea water, 1000 ml and agar, 15 g. The pH was adjusted to 7·6. Liston (1956), using fresh fish, found that counts on medium ZSW were 2–10 times those on medium OHD. Incubation conditions were: 0° (i.e. refrigerator temperature, generally 0° to +3°) for 28 days, 20° for 5 days, 37° for 3 days. Both Petri dish and roll tube counts were made, with duplicate plates or tubes from each dilution examined.

Analysis of the bacterial flora. Various workers (Griffiths, 1937; Wood, 1940; Thjøtta & Sømme, 1943; Shewan, 1949; Georgala, 1958) have shown that the flora of fresh and spoiling fish is composed in the main of bacteria from a limited number of families, viz. the Micrococcaceae, Pseudomonadaceae and Achromobacteraceae, along with small numbers from the Corynebacteriaceae and the Bacillaceae (genus *Bacillus*), and it was considered that the fish box flora would not be markedly different. The bacteria of which these families are formed fall into four groups, each with a characteristic morphology. First, there are Gram-positive to intermediate rods belonging to the family Corynebacteriaceae and the genus *Bacillus*; second, Gram-positive cocci belonging to the family Micrococcaceae; third, a group of slender to filamentous Gram-negative rods belonging to the family Pseudomonadaceae and the genus *Flavobacterium*, and fourth, a group of Gram-negative, plump, blunt-ended rods, oval forms and coccobacilli, characteristic of the genus *Achromobacter*. The colonial appearance is also of assistance in delimiting these groups, since the colonies of the first and last are typically opaque while those of the third are typically translucent. Colonies of *Flavobacterium* are also pigmented. Consequently, to obtain an overall picture of the major bacterial groups present and of their proportions, and also to determine to what extent particular groups of bacteria were being selected by the various counting conditions, single, well isolated colonies were picked off at random from the various count plates of samples taken from dirty boxes, washed boxes and washed and steamed boxes. Generally, twenty colonies were picked off from each count, and the grand total was 1068. With surface colonies, their appearance was noted, as was the presence or absence of pigmentation, and all the isolates were examined morphologically by the Gram stain. On the basis of this examination, they were allocated to one or other of the above morphological groups. There was naturally the possibility of overlapping, particularly with the two groups of Gram-negative rods, but in fact an extended examination of an isolate generally confirmed the preliminary classification.

To obtain more detailed information on the nature of the bacteria isolated, a random selection of the isolates was made and examined further. The 250 strains selected came from counts made on both the media, at all three incubation temperatures, and from several boxes. However, because the 37° counts were low, relatively few of these were represented.

Results

Quantitative aspects

Reliability of results. To obtain reliable results for the effects of washing and steaming, individual boxes had to be sampled before and after washing and again after steaming. As any one area could obviously not be sampled more than once, it was desirable to know the variation to be expected over the surfaces which were normally sampled. Preliminary experiments showed that with both dirty and washed boxes this was usually small, the counts on 8 areas from the sides and bottom of a box before washing ranging from 442×10^6–554×10^6/sample area and those on 8 areas sampled after washing from 333×10^6–1000×10^6. However, after further treatment the variation became somewhat greater.

The effects of different counting conditions. It soon became evident that, irrespective of the sampling method used, the different counting conditions frequently gave quite different results. In particular, the counts at 20°, using both Petri dishes and roll tubes with medium OHD, were always greater than those at 37°, generally about a thousandfold with samples from dirty and washed boxes and a hundredfold or less with samples from steamed boxes or after subsequent standing. This suggests that the bacteria able to grow at 37° had the greater heat resistance.

Other differences between the results obtained with the various counting conditions were not so marked. To bring out the consistent differences of possible practical significance, the results obtained on individual samples with pairs of counting conditions which differed in only one factor were compared in terms of their ratios. The frequency distribution of certain size ranges of these ratios is shown in Table 1. These ranges were chosen because it was considered that a twofold difference, while

Table 1. *The effect of different counting conditions on the apparent bacterial load on commercial wooden fish boxes, as shown by the distribution of ratios of counts obtained with a single factor difference in the conditions*

Comparison* A to B	Other conditions*	Number of samples	Numbers of samples with ratios A:B in the ranges				
			>5	5–2	2–0·5	0·5–0·2	<0·2
A, Petri dishes, vs B, roll tubes	Medium OHD, 20°	22	0	6†	16	0	0
	Medium OHD, 0°	20	2	7	10	1	0
A, medium OHD vs B, medium ZSW	Petri dishes, 20°	22	0	1	17	4	0
	Petri dishes, 0°	20	1	2	17	0	0
A, 20° vs B, 0°	Roll tubes, OHD	69	15	30	23‡	0	1
	Petri dishes, OHD	20	1	5	14§	0	0
	Petri dishes, ZSW	20	3	9	8	0	0

* OHD, heart digest-tap water agar. ZSW, ZoBell sea water based agar.

† One ratio of exactly 2 included. ‡There were 22 ratios >1. §There were 12 ratios >1.

being above the upper limit of variation inherent in the sampling and counting techniques (see above), would be at the lower limit of practical importance while a fivefold difference might be of considerable practical significance.

From Table 1 it is clear that the 20° counts, whether made with roll tubes or Petri dishes with medium OHD or Petri dishes with medium ZSW, were almost always greater than the corresponding 0° counts, and generally very considerably so. Further, when Petri dishes were used the counts tended to be larger than the corresponding roll tube counts, and this was perhaps more marked at 0° than at 20°. However, there were no consistent differences between the two media employed, although occasionally a count on one medium was markedly different from that on the other. This is quite different from the situation with fresh fish where, as mentioned above, medium ZSW consistently gave counts 2–5 times those on medium OHD.

The effects of cleaning procedures. Commercial boxes, obtained after being used on the morning's market, were sampled dirty, i.e. as received from the fish merchants' premises, again after low pressure washing, after steaming, and finally after standing for 48 hr, mainly by the scraping method, though sometimes additional samples were taken by the swabbing method. All the counting conditions previously given were used, but the effects of the various procedures can be well illustrated by the roll tube, medium OHD, 20° results, which are shown in Table 2. Although the boxes appeared considerably cleaner after they had been washed, the pieces of fish gut and clots of slime and blood having been removed, the amount of slime obtained by scraping showed that low pressure washing did not efficiently clean them. This was

Table 2. *The effect of low pressure washing, steaming, and standing for 48 hr, on the bacterial load on commercial wooden fish boxes, as determined by roll tube counts on OHD medium at 20°*

No. of box	Site of sample	Method of sampling	Bacterial load on surface			
			Before washing (10^6/cm^2)	After washing (10^6/cm^2)	After steaming (10^3/cm^2)	After standing (10^3/cm^2)
1	Bottom	Scraping	14·5	29·5	8·6	—*
	Side	Scraping	0·019	29·5	8·4	—
2	Bottom	Scraping	67·0	56·0	21·3	5·0
	Side	Scraping	36·0	116	170	0·57
3	Bottom	Scraping	34·0	42·0	26·0	28·0
		Swabbing	3·6	8·6	0·053	8·2
	Side	Scraping	41·0	30·0	1·0	<0·027
		Swabbing	7·3	9·8	0·43	0·11
4	Bottom	Scraping	13·5	14·6	3·7	—
		Swabbing	3·1	8·8	0·45	—
	Side	Scraping	13·4	27·0	8·9	—
		Swabbing	4·9	8·4	0·27	—
5	Bottom	Scraping	—	11·3	11·0	0·38
		Swabbing	—	0·95	—	0·53
	Side	Scraping	—	10·7	9·8	0·032
		Swabbing	—	4·4	4·8	0·003

* —, not done.

confirmed by the counts, which indeed were frequently considerably higher on the washed than on the dirty boxes, due possibly to redistribution of bacteria present in even larger numbers in the corners and on the edges. Steaming, however, caused a considerable decrease in the bacterial load, at the best to one-thousandth. There was no strong indication of either a general build-up or a decline in numbers during standing.

It was obvious when sampling that swabbing removed only the loose surface slime while scraping removed much more material. This is confirmed by the counts (Table 2) for boxes which were sampled both by scraping and by swabbing. For the dirty and washed boxes the former method gave counts which were generally 2–10 times as large as the latter. Sometimes the differences were even greater after steaming, which may mean that steaming affected mainly the surface of the slime layer.

Table 3. *The effect of high pressure washing, and of subsequent steaming, on the bacterial load on commercial wooden fish boxes, as determined by roll tube counts on OHD medium at 20°*

No. of box	Method of sampling	Bacterial load on surface		
		Before washing (10^6/cm^2)	After washing (10^6/cm^2)	After steaming (10^3/cm^2)
1	Scraping	28	6·1	96
	Swabbing	2·2	5·1	3·3
2	Scraping	12·3	24·6	7·6
	Swabbing	2·7	0·67	3·9
3	Scraping	12·5	1·17	—*
	Swabbing	6·1	1·05	—
4	Scraping	9·1	59	—
	Swabbing	2·0	1·5	—
5	Scraping	8·0	4·8	—
	Swabbing	1·25	6·2	—

* —, not done.

Since low pressure washing was so inefficient, some dirty boxes were cleaned at commercial premises with a commercial high pressure jet, again with cold mains water, and finally steamed. The results obtained with roll tubes and OHD medium at 20° are shown in Table 3. These boxes were not so heavily contaminated as the previous lot. It is also clear that high pressure washing did not effect a consistent reduction. There was again a reduction to one-thousandth on steaming.

Qualitative aspects

Broad classification of all isolates. Table 4 shows the distribution of the isolates among the four previously defined broad groups, according to medium and temperature.

A striking feature is that there were large proportions of Gram-positive rods (Corynebacteriaceae-*Bacillus*) on the plates incubated at 0° and 20°. Such large proportions have not been recorded with fresh or spoiling fish (Shewan, 1949). These bacteria were almost entirely coryneform in morphology and about 75% of them

were markedly pigmented, usually yellow but sometimes orange or pink. The Gram-positive rods isolated from the 37° plates were mainly *Bacillus* species. The low proportions of Gram-positive cocci among the 0° and 20° isolates, though not among the 37° isolates, affords another point of difference from previous findings with fish. Several authors who used incubation at or near 20° have recorded the presence of micrococci on fresh fish in proportions greater than 20% (Stewart, 1932; Wood, 1940; Shewan, 1949), while Dyer (1947) found more than 70%. Others, however, found micrococci to be only a low proportion of the total bacteria on fish (Liston, 1957; Georgala, 1958).

Table 4. *The effect of different counting conditions on the incidence of four groups of bacteria in samples taken from dirty, washed, and washed and steamed commercial wooden fish boxes*

Morphological group	Percentages of total isolates* obtained on					
	Medium OHD† at			Medium ZSW† at		
	0°	20°	37°	0°	20°	37°
Gram-positive rods, mainly irregular morphology	43	54	19	40	44	30
Gram-positive cocci	0·5	2	56	0	3	28
Gram-negative rods, slender, filamentous	29	21	17·5	17	27	33
Gram-negative rods, large, plump, blunt ended	28	23	7	42	25·5	9
	57	44	24·5	59	52·5	42

* The results are based on 12 samples examined on each medium at 0° and 20°, and 3 on each medium at 37°. There were 1068 isolates in all. On OHD there were 239 at 0°, 240 at 20° and 57 at 37°. On ZSW there were 239 at 0°, 239 at 20° and 54 at 37°.

† OHD, heart digest-tap water agar. ZSW, ZoBell sea water based agar.

Table 4 also shows that the counting conditions affected the proportions of the groups of bacteria. The proportions of micrococci were very much higher at 37° than at the other two temperatures, particularly with medium OHD. Considering the two lower temperatures, the highest proportion of Gram-positive rods, mainly coryneforms, was found on medium OHD at 20° and the lowest on medium ZSW at 0°, the difference being 14%. Conversely, the highest proportions of the Gram-negative rods were found at 0° on both media and the lowest on medium OHD at 20°, the difference being 15%. In both cases, the higher values were about one-third greater than the lower ones. At 0° and 20° there were about equal proportions of the two sorts of Gram-negative bacteria on both media, except on medium ZSW at 0°, where the large plump rods were greatly in excess of the slender, filamentous types. As compared with medium OHD at 20° the proportion of large plump rods had risen by over 80%.

The classification of selected strains. Table 5 shows the classification of the 250 strains selected at random for more detailed study, and it is evident that the proportions of the various groups present compare well with the results given in Table 4 for the total isolates from the 20° and 0° counts.

The 137 strains classified as Corynebacteriaceae, various genera of which have been reported as present in fresh or spoiling fish or in sea water (Wood, 1940, 1950, 1953; Dyer, 1947; Liston, 1957; Georgala, 1958), were biochemically heterogeneous although all presented the typical coryneform morphology. Since full descriptions of saprophytic corynebacteria are rare, attempts to relate the present strains to previously described strains isolated from soil, water and marine sources met with little success. These attempts were further complicated by the lack of correlation between the results of the biochemical tests used here in classifying the strains. On the basis of pigment formation (i.e. none, yellow, orange or pink), gelatin liquefaction, changes in litmus milk, nitrate reduction and sugar fermentations, 31 different groups were obtained, of which only 4 contained more than 10 strains. There are few descriptions in the literature of non-pigmented coryneform bacteria isolated from soil and water, and no reports of such organisms have been found in the literature dealing with marine and fish bacteriology. It is consequently possible that many of the non-pigmented strains isolated, 50 in all, will be found on further examination to be new species. Many of the yellow and orange strains were similar to isolates from fish described by previous workers, particularly Aschehoug & Vesterhus (1943), Thjøtta & Sømme (1943) and Wood (1950), although others would appear not to have been described.

Table 5. *The classification of a random selection of* 250 *strains isolated from commercial wooden fish boxes and studied in detail*

Classification	Incidence of groups	
	No.	%
Corynebacteriaceae	137	54·8
Achromobacteraceae		
Achromobacter	46	18·4
Flavobacterium	8	3·2
Pseudomonadaceae	36	14·4
Micrococcaceae	17	6·8
Unclassified	6	2·4
Totals	250	100

The 46 strains of *Achromobacter* could be divided into ten groups on the basis of reaction in litmus milk, fermentation of sugars and reduction of nitrate; a few of the strains were motile or liquefied gelatin. Many of these groups were similar to species previously described from marine sources, such as the *Achr. album pestifer* group recognized by Harrison, Perry & Smith (1926) and subsequent workers, and the acid-producing *Achr. acidum* group recognized frequently in materials from marine fish. Those groups which produced alkali in litmus milk, which would be classified as *Alcaligenes* according to Bergey's Manual (Breed, Murray & Smith, 1957) and indeed appeared similar to *Alcaligenes metalcaligenes*, were very similar to strains frequently isolated from fish by Liston (1955) and Georgala (1957*b*).

All the 8 strains of *Flavobacterium* were Gram-negative non-motile small rods. Their colonies, of various shades of yellow, were transparent to translucent. This

genus has frequently been reported to form an appreciable portion of the flora of marine fish, both fresh and spoiling, and the strains examined here were similar to many previously described by workers in this field.

The 36 strains classified in the family Pseudomonadaceae fell into five well defined groups, the first four being within the genus *Pseudomonas* in that they dissimilated glucose aerobically, or not at all. The first group comprised 15 non-pigmented strains which had very little apparent biochemical activity. Sugars were rarely attacked (as shown by conventional methods), gelatin was not liquefied, litmus milk was, at the most, turned slightly alkaline or acid, and nitrate was occasionally reduced. The second group, consisting of 8 strains, was characterized by the production of a brown diffusible pigment and was biochemically inactive except for ability to liquefy gelatin and produce alkaline peptonization in litmus milk. Similar strains have been isolated from fresh fish by Liston (1955), who found that they had, on first isolation, a marked preference for sea water based media. They would thus appear to be typically marine by ZoBell's definition (ZoBell, 1946), and may constitute a new species. Four strains which produced a green diffusible fluorescent pigment formed a third group. According to Bergey's Manual (Breed, Murray & Smith, 1957) these should be classified as *Ps. ovalis* (one strain), *Ps. pavonaceae* (two strains) and *Ps. fluorescens* (one strain). The fourth group consisted of one strain which formed yellow translucent colonies. This strain, which was inactive biochemically, does not appear to have been previously described. The fifth group, which was initially allocated to the genus *Aeromonas*, consisted of 8 strains characterized by the ability to ferment many sugars with the production of gas. All were Voges-Proskauer positive. A more critical examination of the flagellation of these strains, however, showed that this could vary within a single culture from a single polar flagellum, through several polar flagella, to several, generally two to six, peritrichous flagella. It would thus appear that these organisms should properly be classified as paracolons, within the family Enterobacteriaceae, members of which have previously been reported to be present on fresh water and marine fish (Griffin & Snieszko, 1951; Venkataraman & Sreenivasan, 1953*a*,*b*).

Of the 17 strains of *Micrococcus*, six were pigmented yellow, liquefied gelatin and reduced nitrate. They had varied reactions in litmus milk. They were similar to the *Mic. flavus* group of Wood (1952). Nine non-pigmented strains with varied biochemical reactions were similar to Wood's *Mic. candidus* group, while 2 strains were orange-pigmented with a sarcinal morphology.

The 6 strains in the '*Bacterium*' group were, for a number of reasons, impossible to classify in any of the preceding genera.

Discussion

It is now abundantly clear that spoilage of wet white fish is due mainly to bacterial action and, at least on board the trawler, is due to the indigenous psychrophilic flora of the exterior and interior of the fish when it is caught. Probably different bacterial groups will predominate at different stages of spoilage (Report, 1956*a*). Any later additions of similar psychrophilic bacteria from the environment will increase the spoilage potential, and further, when the fish is held on land at higher temperatures

and is being processed, contamination by mesophilic terrestrial bacteria may be important.

The internal surfaces of one type of wooden box used to hold fish unloaded from trawlers and to transport it to wholesalers' premises have been shown to carry large bacterial loads, of the order of 10^7–10^8/cm^2, which are not markedly reduced by hosing with cold mains water, the usual commercial box washing procedure. Nevertheless, it is considered doubtful whether even such heavily contaminated boxes would cause enhanced spoilage of fish put into them, because of the short time it spends therein, generally less than 12 hr. Wood, however, is used almost exclusively for the walls of trawlers' fish rooms and for the pound boards which separate the fish room into compartments. Also, in trawlers much fish is held in boxes similar to those examined in the present work. If, as seems likely, the wood surfaces on trawlers are equally heavily contaminated and as unresponsive to the cleaning methods now used, there is a distinct possibility of a deleterious effect on the fish because of the time of contact involved, which will frequently be 8–10 days and may extend to 16 days. Indeed, it has recently been shown that fish stored in new wooden boxes for up to 12 days at an ambient temperature of 2·5° are organoleptically in better condition than fish stored under the same conditions in dirty boxes (Report, 1956*b*). Further, recent Canadian work has shown that a peculiar condition of fish which develops on trawlers and is known as 'bilgyness' is due to contact with heavily contaminated wooden surfaces (MacCallum, 1955), and there is reason to believe that a similar condition causing concern in the British fishing industry is due to the same cause.

Why there should be such large proportions of coryneform bacteria on boxes is not at present understood. Presumably the main source of the bacteria on boxes is the skin of previous fish; but this would not provide coryneform bacteria in the observed proportions, although some would almost certainly be present. It is also unlikely that the counting techniques used were selecting this group, since they did not do so concurrently with samples from fish. It is possible that the coryneforms were being favoured in the slime on the boxes at the expense of the pseudomonads and achromobacters by some unknown factor: but there is no evidence for this, and a number of strains of the coryneforms showed no evidence of cellulase activity when tested against filter paper.

In this investigation, Petri dishes tended to give higher counts than did roll tubes. This accords with the results obtained by Clegg *et al.* (1951) with milk samples, but other investigators (Wilson, 1922; Snyder, 1947) have observed the contrary. The disagreement is possibly due to differences in the apparatus and technique used.

Generally, there was close agreement between the results obtained with the two media, though there was a great proportionate increase, almost 100%, in the achromobacters in counts on medium ZSW at 0° as compared with 20°. It is possibly significant that these organisms are primarily associated with marine sources. This close agreement was at first sight surprising since, concurrently with these investigations, the same media and counting techniques were being used for samples from fish, and medium ZSW was consistently giving 2–5 times as many colonies as medium OHD. Probably, the explanation is that coryneform bacteria comprised 40–50% of

the total flora of the box samples, but only 5–20% of that of fish slime. This explanation is supported by the fact that the great majority of the isolates grew equally well on both the media.

The finding that incubation at 20° gave larger counts than incubation at 0° is in agreement with the general experience with material from marine sources, that maximum counts are obtained at or around 20°. In the present work, almost all the strains isolated at 0° grew better at 20°, but strains isolated at 20° frequently did not grow at 0°. The 37° counts were always much lower than those at 20°, and it was confirmed that few of the cultures isolated at 20° would grow at 37°. The tendency for the difference between the 20° and the 0° counts to be larger when roll tubes were used cannot at present be explained, but if the effect is real it could be of considerable practical importance. If different bacterial populations were being selected an erroneous picture of the flora would be given.

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A STUDY OF THE BACTERIA ASSOCIATED WITH THE ROOTS OF SUBTERRANEAN CLOVER AND WIMMERA RYE-GRASS

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SUMMARY: A study of the bacteria from the surfaces of roots of subterranean clover (*T. subterraneum* L.) and Wimmera rye-grass (*L. rigidum* Gaud.) revealed that 21 genera were represented among the isolates from clover and 16 genera among those from rye-grass. Bacteria showing branched forms predominated and accounted for 63% of the 151 clover isolates and 78% of the 167 grass isolates. Most of these were identified as *Arthrobacter*, but from clover a significant proportion were *Nocardia*-like types. Members of the genera *Mycoplana*, *Micromonospora*, *Mycobacterium*, and *Mycococcus* were also identified among the branching forms.

Although the soil had been inoculated with effective rhizobia and the clover plants were effectively nodulated only one of the 318 isolates was capable of nodulating subterranean clover. The majority of the isolates were chromogenic and Gram-negative, produced acid from glucose and ammonia from peptone, were catalase-positive and grew best aerobically. Approximately half the isolates liquefied gelatin and produced hydrogen sulphide from peptone.

In the reviews by Katznelson, Lochhead & Timonin (1948) and Clark (1949) many examples are given of the selective stimulation of the soil microflora by plant roots. In general, the rhizosphere microflora has been characterized on three bases: morphology and staining (Clark, 1940; Lochhead, 1940; Webley, Eastwood & Gimingham, 1952; King & Wallace, 1956; Rovira, 1956); physiological and biochemical characteristics (Starkey, 1931; Lochhead, 1940; King & Wallace, 1956; Katznelson & Rouatt, 1957), and nutritional requirements (Lochhead & Chase, 1943; Wallace & King, 1954; Lochhead & Rouatt, 1955; Gyllenberg, 1957). But despite the considerable amount of study on the characteristics of the rhizosphere microflora no attempt has been made to classify all the isolates into their respective genera.

The distinction between the members of the genus *Bacillus* and non-sporing bacteria among isolates from the rhizosphere has been made by Krassilnikov (1934), Clark (1940), Lochhead (1940), Clark & Smith (1949), Gyllenberg (1955) and Rovira (1956), while Clark (1940) also distinguished the *Pseudomonas* group on the basis of their fluorescence. The stimulation of the genus *Mycoplana* (motile) in preference to the genus *Corynebacterium* (non-motile) was reported by Lochhead (1940). Lochhead (1948) also demonstrated a higher proportion of chromogenic bacteria related to *Bacterium globiforme* in isolates from the rhizosphere than among those from control soil. In a study of the microflora of the rhizospheres and the root surfaces of plants from coastal sand dunes Webley *et al.* (1952) distinguished the *Bacillus*, *Corynebacterium*, *Mycobacterium* and *Nocardia* groups.

The work described in this paper was initiated to obtain information on the

bacterial genera associated with the roots of subterranean clover and Wimmera rye-grass. Information on the staining, morphological, and physiological characteristics of the isolates obtained during this investigation is also reported.

Materials and Methods

Method of isolation. The roots of plants of subterranean clover (*Trifolium subterraneum* L.) and Wimmera rye-grass (*Lolium rigidum* Gaud.), grown for 15 weeks in a sand-loam mixture (inoculated with a suspension of clover rhizobia), were carefully washed in sterile water. These roots were transferred to conical flasks containing 100 ml of Ringer's solution and glass beads and shaken vigorously for 15 min on a 'Microid' flask shaker. Aliquots from the serial dilutions prepared from the initial suspensions were transferred to Petri dishes and plates poured with the yeast extract-peptone-soil extract agar (YPS) of Bunt & Rovira (1955). The plates were incubated at 26° for 14 days, those showing a suitable number of discrete colonies were selected and then each colony from such plates or sectors thereof was subcultured into yeast extract-peptone-nitrate broth (see below) and on to glucose-yeast extract-apatite agar (Sperber, 1958). Each resulting culture was checked for purity in the former medium and maintained on a YPS agar slope. Of the 318 colony pickings, 29 proved to be mixtures of two or three types which were separated by plating.

Media, and incubation and examination of cultures

Mineral salts base. This contained K_2HPO_4, 0·4 g; $(NH_4)_2\ HPO_4$, 0·5 g; $MgSO_4.7H_2O$, 0·05 g; $MgCl_2$, 0·1 g; $CaCl_2$, 0·1 g, and $FeCl_3$, 0·01 g in distilled water, 1 l.

Yeast extract-peptone-nitrate broth. This consisted of yeast extract, 2·0 g; peptone, 2·0 g, and KNO_3, 0·5 g in mineral salts base without $(NH_4)_2HPO_4$, 1 l. It was used to detect the following products after incubation at 26° for 7 days; hydrogen sulphide (with lead acetate paper), ammonia (with Nessler's reagent) and nitrite (with the α-naphthylamine-sulphanilic acid-acetic acid reagent).

Glucose broth. This contained KH_2PO_4, 0·4 g; $CaCl_2$, 0·1 g; $MgSO_4.7H_2O$, 0·25 g; glucose, 10 g, and yeast extract, 0·5 g, in distilled water, 1 l. The pH was adjusted to 7·0 and a mixture of equal parts of saturated aqueous bromo-cresol purple and cresol red was added (4 ml/l. of medium). Durham fermentation tubes were used, and cultures were incubated at 26°: the pH values were recorded after 1, 2, 4, 7 and 14 days.

Alkylamine medium. For this, ethanolamine was added to tubes of sterile mineral salts base to give a final concentration of 0·5% (v/v).

Alginic acid medium. This comprised 0·2% (w/v) of sodium alginate in the mineral salts base.

Phenol medium. This contained 0·05% (w/v) phenol in a mineral salts solution (Gray & Thornton, 1928).

Chitin agar. A 1% (w/v) suspension of chitin (British Drug Houses, Ltd.), prepared by the method of Campbell & Williams (1951), was incorporated in mineral salts base with 1·5% agar. One set of plates was poured with chitin agar only and a second set was poured as a layer over solidified YPS agar. Cultures were spotted

on segments of the plates, which were examined for clearing of the chitin at intervals during incubation at 26° for 14 days.

Yeast extract-mannitol agar (J. R. Harris, pers. comm.). A solution was made of K_2HPO_4, 1·0 g; $MgSO_4.7H_2O$, 0·2 g; NaCl, 0·1 g, and $CaCl_2$, 0·1 g, in 1 l. of filtered tap water. To this was added yeast extract (Difco), 1·0 g ; mannitol, 10 g; $CaCO_3$, 1·5 g; ferric citrate (0·6%), 2·5 ml, and agar, 10 g.

Sauerkraut-sucrose-glycerophosphate medium (J. R. Harris, pers. comm.). This contained sucrose, 10 g; $MgSO_4.7H_2O$, 0·3 g; NaCl, 0·2 g; KCl, 0·1 g; calcium glycerophosphate, 1·0 g; NH_4NO_3, 0·25 g; ferric citrate (6%), 0·5 ml; sauerkraut juice, 20 ml, and distilled water, 1000 ml.

Lactose broth. Difco 'Purple broth base' with 1% of lactose was used and incubated at 26° for 40 days.

Ulrich milk (Ulrich, 1944). The reactions were recorded after incubation for 14 days at 26°.

Gelatin. A solution containing 15% (w/v) of gelatin in tap water was used. Cultures were incubated at 20° and the results recorded after 14 and 28 days. Any action, from slight (forming dry cavities) to complete liquefaction of a 10 ml portion, was recorded as positive.

Action on apatite. This was detected by clearing around the inoculum within 14 days at 26° on the glucose-yeast extract-apatite agar of Sperber (1958).

Oxygen requirements. The ability of the isolates to grow and to dissolve apatite anaerobically was tested by inoculating on to glucose-yeast extract-apatite agar and incubating for 14 days in a McIntosh & Fildes anaerobic jar.

Clover nodulation. All the isolates were inoculated in triplicate on to ten-day old subterranean clover plants growing in nitrogen-free plant nutrient agar. The plants were examined for nodules at intervals over 28 days.

Heat resistance. All cultures were tested by heating YPS broth at 80° for 10 min. Any cultures which showed growth after this treatment were examined microscopically for spores.

Motility. Growth from an 18–24 hr slope culture (incubated at 26°) on semi-solid agar was suspended in Ringer's solution, incubated at 26° for 10–15 min and then examined under phase-contrast illumination.

Fluorescence. Cultures grown on yeast extract-mannitol agar (see above) at room temperature for 7 days were examined in ultraviolet light.

Catalase. The catalase reaction was tested with 10-vol. H_2O_2 on a 24 hr slope culture on YPS agar.

Staining methods

Simple stain with safranin, 1% aqueous. This was initially done on the same material as was used for the Gram stain but subsequently organisms grown in sauerkraut-sucrose-glycerophosphate broth were used. Smears were prepared from cultures 6–72 hr old.

Gram stain. This was done on the first day that the organisms showed visible growth on YPS agar.

Giemsa stain. Wet smears were fixed in osmic acid vapour for 1 min and then

stained for 1 hr with dilute Giemsa stain (one part Giemsa to 30 parts of phosphate buffer, pH 6·8).

Congo red cell wall stain. The CPC-congo red method of Dyar (1947) was used, but the methylene blue counter-stain was omitted.

Flagella stains. The methods of Gray (1926), Fisher & Conn (1942) and Leifson (1951) were used.

Acid-fast stain. Acid fastness was tested with 20% H_2SO_4, which was found suitable for *Mycobacterium phlei.*

Photography. Photomicrographs were taken on Ilford 7×5 in. R.40 plates using the Officine Galileo Universal camera microscope with either an Ilford 202 (orange) or Ilford 404 (green) filter and phase-contrast optics.

Results

Generic identification of the isolates. The 151 isolates from subterranean clover contained representatives of 21 genera (or groups of genera as later discussed) and the 167 isolates from Wimmera rye-grass embraced 16 genera (Table 1). Notwithstanding the range of genera encountered, branched forms predominated and accounted for 63% of the clover and 78% of the grass isolates. Most of these were identified as *Arthrobacter* (Plate 1, a–h), but from clover a significant proportion was *Nocardia*-like (Plate 1, 1; Plate 2, a–c).

Table 1. *The allocation to genera of* 151 *organisms isolated from the roots of clover and* 167 *from the roots of Wimmera rye-grass*

Genus	No. of isolates		Genus	No. of isolates	
	Clover	Rye-grass		Clover	Rye-grass
Branched forms			Non-branched forms		
Arthrobacter	62	116	*Achromobacter*	4	3
Nocardia (Gram −ve)	19	5	P.Al.A.*	1	5
Mycoplana	8	9	X.F.*	3	3
Mycobacterium	3	0	*Flavobacterium*	4	1
Micromonospora	3	0	*Serratia*	2	3
Mycococcus	0	1	*Sarcina*	3	2
			Alginomonas	4	0
			Bacillus	3	0
Non-branched forms			*Micrococcus*	1	1
P.X.F.E.*	8	4	*Alcaligenes*	1	0
P.A.*	11	2	*Rhizobium*	1	0
X.Al.F.*	6	4	*Beneckia*	1	0
Brevibacterium	3	7	Yeast	0	1

* Because the final identification of some organisms could not be completed due to unsatisfactory flagella staining they were placed in the following groups, according to Skerman's key (Breed *et al.* 1957):

P.X.F.E., *Pseudomonas, Xanthomonas, Flavobacterium, Erwinia* group—acid from lactose.

P.A., *Pseudomonas, Achromobacter* group—no acid from lactose, motile, acid from glucose, not pigmented.

X.F., *Xanthomonas, Flavobacterium* group—no acid from lactose, motile, acid from glucose, colonies yellow.

P.Al.A., *Pseudomonas, Alcaligenes, Achromobacter* group—no acid from lactose, motile, no acid from glucose, not pigmented.

X.Al.F., *Xanthomonas, Alcaligenes, Flavobacterium* group—no acid from lactose, motile, no acid from glucose, colonies yellow.

In using Skerman's Key of Identification in Bergey's Manual, 7th ed. (Breed *et al.* 1957) the genera *Pseudomonas*, *Xanthomonas*, *Flavobacterium*, *Erwinia*, *Achromobacter* and *Alcaligenes* were not differentiated on account of the unsatisfactory results of the flagella staining. This led to these genera being grouped on the basis of their common reactions with lactose and glucose, pigmentation and motility, as set out in Skerman's Key. Where identification did not depend on the arrangement of flagella some organisms could be placed in one of the genera *Achromobacter*, *Flavobacterium* or *Alcaligenes*.

It is interesting to note that only one representative of the genus *Rhizobium* (clover-nodulating) was found among the isolates in spite of initial inoculation of the soil with clover rhizobia and the abundance of effective nodules on the clover roots from which the isolates were obtained.

Four isolates from clover which possessed the ability to utilize alginic acid as the sole source of carbon were placed in the genus *Alginomonas*. An organism from clover which dissolved chitin when the chitin agar was layered over YPS agar was identified as *Beneckia*. It did not dissolve chitin when chitin agar alone was used.

Table 2. *The morphological characteristics and Gram-staining of* 151 *bacteria isolated from clover roots and* 167 *from rye-grass roots*

Gram reaction	Morphology	Numbers of organisms from	
		Clover	Rye-grass
Positive	rods	4	8
	cocco-bacilli	1	0
	cocci	4	4
	branched forms	6	32
Negative	rods	39	24
	cocco-bacilli	8	5
	cocci	0	0
	branched forms	89	94

Staining characteristics and morphology. Of the clover isolates 90%, and of the rye-grass isolates 73%, were Gram-negative (Table 2), but the grass roots supported a considerably larger number of Gram-positive branched forms.

Physiological and biochemical properties. As shown in Table 3, the majority of the organisms from both sources produced acid from glucose within 14 days but none produced gas. The glucose medium used is very sensitive to pH changes, more so than a medium buffered by beef extract or peptone components. The glucose broth resembled the glucose-yeast extract-apatite agar medium except in the phosphate component, soluble phosphate in place of apatite. This permitted a study of the relationship between acid production and the solution of apatite. The latter was dissolved by 27% of the Gram-positive and 46% of the Gram-negative clover isolates but by only 27% of the Gram-positive and 30% of the Gram-negative isolates from grass. As found previously by Sperber (1958) the degree of solubilization was not proportional to the fall in pH.

Most of the organisms from both sources released ammonia from amino acids, were

catalase-positive and grew best aerobically, but less than half released hydrogen sulphide from peptone or reduced nitrate to nitrite. Several of the cultures fluoresced under ultraviolet light when grown on yeast-mannitol agar and some of these released fluorescent substances into the medium. None of the isolates caused the disintegration of filter paper when growing in 0·5% peptone water.

Table 3. *The physiological and biochemical properties of* 151 *bacteria isolated from clover roots and* 167 *from rye-grass roots*

Test	Reaction	Numbers of isolates from			
		Clover		Rye-grass	
		15 Gram +	136 Gram −	44 Gram +	123 Gram −
Glucose pH	<5·2	7	81	26	58
	5·2–6·0	4	25	6	20
	>6·0	4	30	12	45
H_2S production	+	5	46	13	52
	−	10	90	31	71
NH_3 production	+	10	104	26	95
	−	5	32	18	28
NO_2^- production	+	6	39	9	27
	−	9	97	35	96
Gelatinase	+	8	74	10	52
	−	7	62	34	71
Catalase	++	6	20	14	14
	+	7	74	18	67
	−	2	42	12	42
Anaerobic growth	++	2	3	1	8
	+	8	73	20	41
	−	5	60	23	74
Solution of apatite	++	1	21	3	7
	+	3	41	9	30
	−	11	74	32	86
Chromogenesis	+	4	78	33	87
	−	11	58	11	36
Motility	+	5	88	10	86
	−	10	48	34	37
Fluorescence	+	0	8 (2†)	4 (3†)	9 (2†)
	−	15	128	40	114

† The bracketed figures are the numbers of fluorescent organisms producing a fluorescent exudate.

A closer study of the biochemical and physiological properties of the branched organisms (Table 4) revealed differences between the mycoplanas, arthrobacters, and nocardias. The mycoplanas were less active in the production of acid from glucose, in nitrate reduction and in solution of apatite than the arthrobacters and nocardias. Among the branched isolates from clover roots the nocardias were more active than the arthrobacters in acid production from glucose, ammonification, and gelatin digestion and contained more chromogenic types, but they contained fewer hydrogen sulphide producers, motile and fluorescent types.

Table 4. *The physiological and biochemical properties of bacteria showing branched forms isolated from roots of clover and rye-grass*

Test	Reaction	Numbers of organisms allocated to the genera											
		Arthro-bacter		*Nocardia**		*Myco-plana*		*Myco-bacterium*		*Micro-monospora*		*Myco-coccus*	
		Clover (62)	Grass (116)	Clover (19)	Grass (5)	Clover (8)	Grass (9)	Clover (3)	Grass (0)	Clover (3)	Grass (0)	Clover (0)	Grass (1)
Glucose	pH <5·2	36	66	18	1	0	0	2	0	1	0	0	1
	pH 5·2–6·0	13	20	1	0	1	0	0	0	1	0	0	0
	pH >6·0	13	30	0	4	7	9	1	0	1	0	0	0
H_2S production	+	23	50	2	2	6	3	1	0	2	0	0	0
	—	39	66	17	3	2	6	2	0	1	0	0	1
NH_3 production	+	49	88	19	4	8	7	2	0	3	0	0	0
	—	13	28	0	1	0	2	1	0	0	0	0	1
NO_2^- production	+	16	29	7	2	2	0	0	0	1	0	0	0
	—	46	87	12	3	6	9	3	0	2	0	0	1
Gelatinase	+	28	46	18	2	3	2	2	0	2	0	0	0
	—	34	70	1	3	5	7	1	0	1	0	0	1
Catalase	++	8	27	4	0	1	1	0	0	0	0	0	0
	+	38	51	13	1	6	7	3	0	2	0	0	1
	—	16	38	2	4	1	1	0	0	1	0	0	0
Anaerobic growth	++	1	5	0	1	0	1	0	0	0	0	0	0
	+	37	43	11	1	6	5	1	0	2	0	0	0
	—	24	68	8	3	2	3	2	0	1	0	0	1
Solution of apatite	++	11	8	3	0	0	0	0	0	0	0	0	0
	+	19	28	13	2	3	1	2	0	2	0	0	0
	—	32	80	3	3	5	8	1	0	1	0	0	1
Chromogenesis	+	31	89	18	5	3	6	1	0	2	0	0	1
	—	31	27	1	0	5	3	2	0	1	0	0	0
Motility	+	41	64	4	3	7	8	2	0	2	0	0	0
	—	21	52	15	2	1	1	1	0	1	0	0	1
Fluorescence	+	4	11(5†)	0	0	0	0	1	0	1	0	0	0
	—	58	105	19	5	8	9	2	0	2	0	0	1

* Gram-negative. † The number of fluorescent organisms producing a fluorescent exudate.

Discussion

In the past the identification of soil bacteria has been hampered by the lack of a key taking into account their Gram variability, pleomorphism and biochemical inertia. As the organisms on plant roots originate mainly from the indigenous soil and seed coat microfloras it is probable that their identification will present many of the problems experienced with soil bacteria. However, the more rapid growth and greater biochemical activity of most root surface isolates should facilitate their identification. The recent publication of a revised key for the determination of bacterial genera by Skerman in the 7th edition of Bergey's Manual (Breed *et al.* 1957) has improved the chances of making correct identifications of soil isolates. In this key the Gram variability is covered by placing many genera in both the Gram-positive and -negative sections, while the early separation of branched forms simplifies identification. However, considerable difficulty is still experienced in identifying unbranched organisms which do not utilize specific substrates, e.g. chitin, phenol, alkylamines, alginic acid, etc. The emphasis placed on the arrangement of flagella as a character distinguishing *Pseudomonas* and *Xanthomonas* from *Erwinia*, *Achromobacter*, *Alcaligenes*, and *Flavobacterium* is a weakness of the key in view of the failure of present flagella staining techniques to give reliable results. Possibly the use of

such characteristics as the production of acid from sugars under aerobic and anaerobic conditions, pigmentation, and litmus milk reaction could be used to separate these genera. The necessary omission of the non-pathogenic *Agrobacterium* spp. as a separate genus from the key is unfortunate. It is felt that the root surface could provide a suitable habitat for *Agr. radiobacter* and that use could be made of its ability to turn litmus milk and mannitol-glycerophosphate brown to distinguish it from the species of *Alcaligenes* and *Rhizobium* likely to occur in soil or on roots.

The separation of the branched forms from the remainder raises the problem of the most satisfactory method of studying morphology. In the initial examination of the isolates used in this study the smears were prepared from 24–48 hr cultures grown in YPS agar, and although some pleomorphic and filamentous forms were observed these were rare, so that from this initial survey only a few of the isolates would have been placed in *Arthrobacter* or associated genera. However, with the use of sucrose-sauerkraut-calcium glycerophosphate, careful preparation of the smears after 24 and 48 hr at 26°, and simple staining with safranin, it was possible to detect many more branching forms. This emphasizes the importance of a standardization of media and techniques in work of this kind; the use only of 24 hr cultures from YPS agar slopes in this study would have resulted in the conclusion that the majority of the isolates were short Gram-negative rods.

The finding of so many bacteria showing branching has enabled a close study to be made of the many different types. In Skerman's key the criterion for *Arthrobacter* is that 'ultimately the rods transform completely to cocci', but in many of the isolates this characteristic proved to be variable and to depend on the medium and incubation period. The tendency to become granular and to fragment is shown in Plate 1, a and b, in which the 6 hr culture shows extreme pleomorphism and branching, while in the 24 hr culture, although several branched forms are still apparent, considerable fragmentation has occurred. In contrast, c and d in Plate 1 show 24 and 48 hr cultures of another organism in which only occasional coccoid cells can be seen. The occurrence of coccoid, branched, and filamentous forms in 24 and 48 hr cultures is shown in Plate 1, e–h, but complete fragmentation was seldom observed under the conditions of study. In spite of this the authors feel that there is justification for including any non-acid fast, pleomorphic, branched forms not utilizing phenols and not developing conidia on side branches, in the genus *Arthrobacter*.

Skerman states that if limited true branching occurs in *Arthrobacter* then the "dividing line between *Arthrobacter* and *Nocardia* becomes very slim". We have made the distinction between *Nocardia* and *Arthrobacter* on the assumption that nocardias produce long, slender, branched filaments with parallel sides, which break up into regular coccoid rods (Plate 2, a–c) in contrast to the irregular, branching rods of the arthrobacters. It should be noted, however, that the isolates here designated *Nocardia* were Gram-negative; this is not compatible with the description of this genus in Bergey's Manual, and hence it might be preferable at this stage to refer to them as *Nocardia-like* arthrobacters. For convenience they will be referred to as *Nocardia* in this study. The difficulty in distinguishing the two groups is illustrated in Plate 1, g and h, with organism 311, in which long filaments with parallel sides were occasionally found whereas its usual morphology was pleomorphic

in nature. Another similarity was the 'star' arrangement of cells (Plate 1, k and l) commonly found in *Nocardia* and occasionally in *Arthrobacter*. In Plate 1 l, several stages in this 'star' formation are apparent, commencing as one or two filaments coming from a central coccoid cell and developing into larger clusters. This germination of 'cystites' or 'coccoids' is similar to that reported by Jensen (1934), Conn & Dimmick (1947) and Sundman (1958). The development of the 'star' arrangement following the germination of the coccoid cell differs from the aggregation of cells which occurs during a stage in the sexual cycle reported for *Agrobacterium* (Stapp, 1942) and for *Rhizobium* (Heumann, 1956).

All the branching organisms which utilized phenol were designated *Mycoplana*, although in morphology they were similar to *Arthrobacter* (Plate 2, d, g and h). Cells from a 72 hr culture of *Mycoplana* showed dichromatism when stained with Giemsa (Plate 2, e) due to variations in the basophilic properties of the cells. This indicates possible differences in nucleoprotein content or distribution thereof, and hence cells which stained differently could have been at different stages of a life cycle. The CPC-congo red cell wall stain also showed dichromatism among the cells (Plate 2, f), but in this case the cells which stained intensely corresponded to the short rods which stained only lightly with Giemsa (Plate 2, e).

Several of the organisms which showed branching were identified as *Mycococcus* and *Micromonospora* on morphology alone, while all acid fast isolates were placed in the genus *Mycobacterium*. In one of the three organisms identified as *Micromonospora* short side branches or conidiophores each carrying one or several conidia were found (Plate 2, k and l). Production of these conidia apparently required specific cultural conditions and special care in the preparation of the smear, as they were observed only in some Giemsa stained preparations.

In their morphology, many of the cultures isolated from the root surfaces resembled those described and illustrated by Krassilnikov (1934), Jensen (1934) and Topping (1937). However, as the isolates described by these authors were obtained from non-rhizosphere soil, the fact that similar organisms constituted the majority of the isolates from the root surface indicates the strong selective influence of plant roots on these types. Lochhead (1940, 1948) reported little difference in the incidence of the *Bacterium globiforme* group (now *Arthrobacter* spp.) among isolates from control soil and from the rhizosphere, although there was a preferential stimulation of motile, branching forms and chromogenic types in the rhizosphere. Webley, Eastwood & Gimingham (1952) found that 82% of the isolates from the surfaces of the roots of *Agropyron junceum* and 61% of those from *Ammophila arenaria* had affinities with *Corynebacterium*, *Mycobacterium* or *Nocardia* or showed mycelial forms, while only 40% of the isolates from non-rhizosphere sand belonged to these groups. Rhizosphere samples showed values intermediate between those of the root surface and the open sand.

The results of Clark (1940) are often quoted to illustrate the abundance of the *Pseudomonas* group in the rhizosphere, but closer examination of these results reveals that only one-tenth of the isolates could be accounted for in identifiable groups, including the fluorescent types. Clark commented that "the majority of cultures were coccoidal forms or variable rods that assume a coccoid appearance in older

subcultures". Hence it would be more accurate to conclude that *Pseudomonas* constitutes a minor group in the rhizosphere and that the organisms of variable morphology (probably belonging to the arthrobacters) predominate.

While it must be admitted that it has not been possible confidently to establish the genus of many of the non-branching bacteria, this study has shown that it is branching types which predominate on the root surfaces of subterranean clover and Wimmera rye-grass. In studying branching organisms it is important to specify the medium from which morphology has been studied. In our experience the sauerkraut-sucrose-glycerophosphate medium was the most satisfactory.

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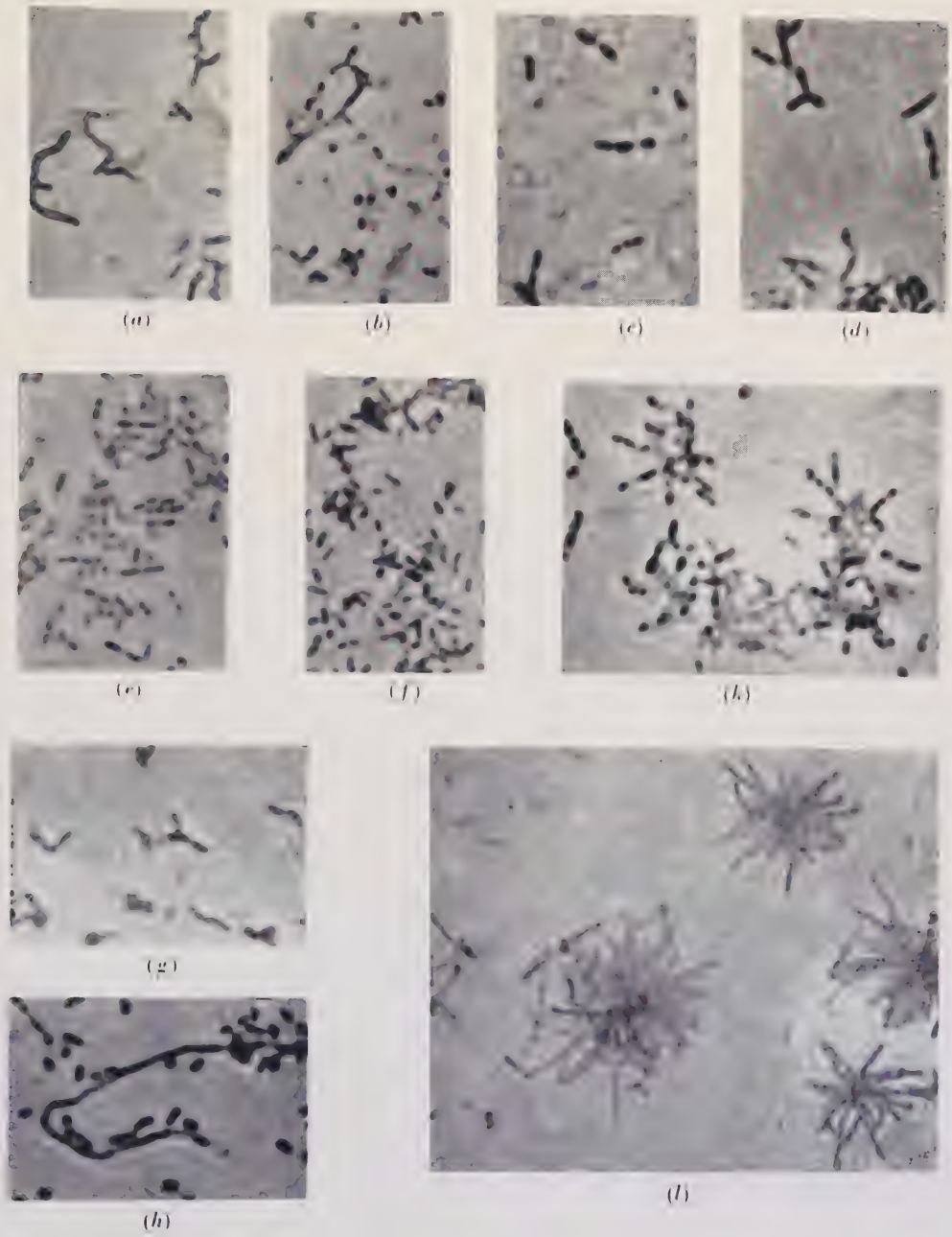
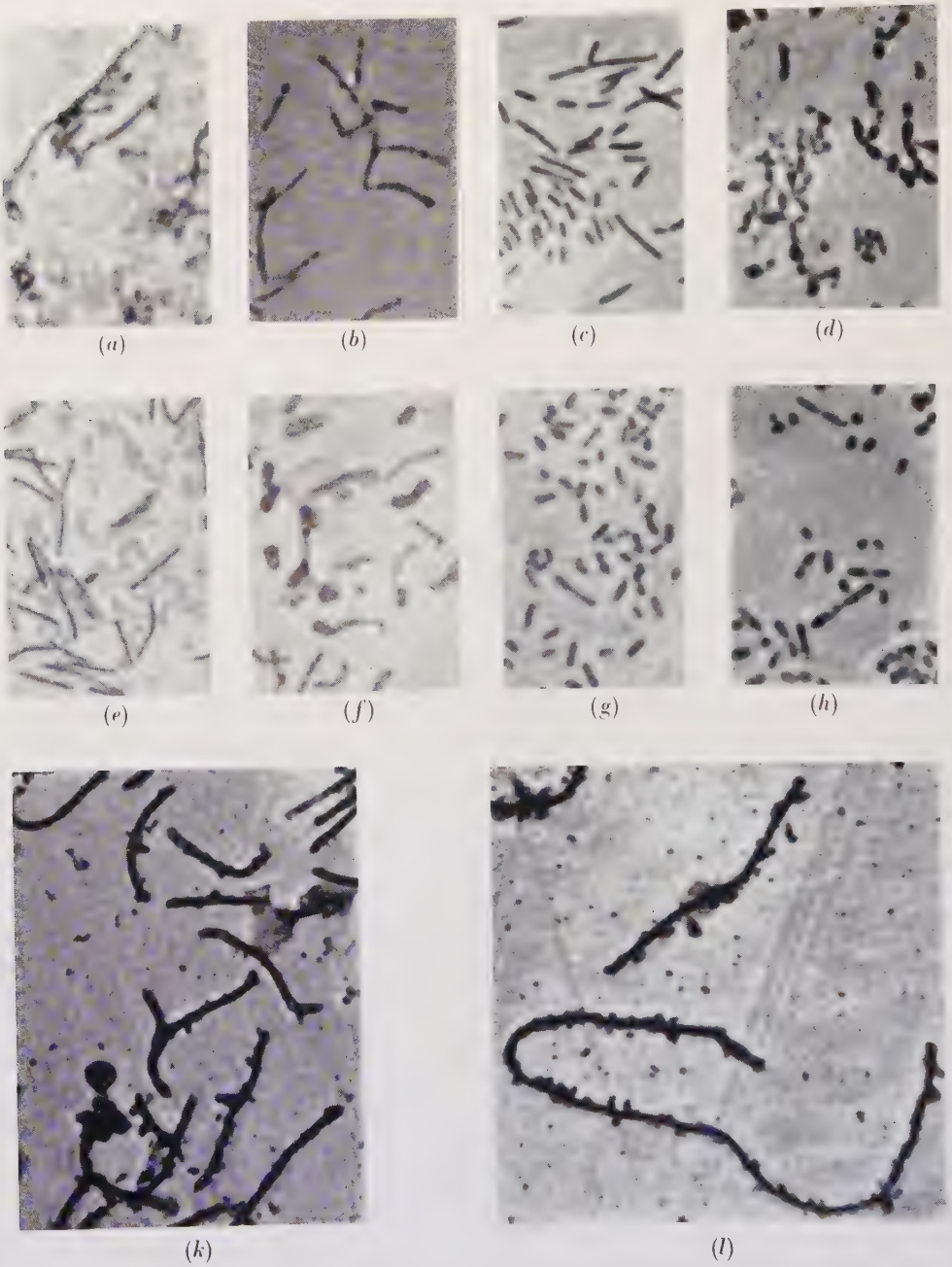


Sperber, Joan I. & Rovira, A. D. — A Study of the Bacteria associated with the Roots of Subterranean Clover and Wimmera Rye-grass. Plate 1



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Explanation of Plates

Plate 1. Cultures in sauerkraut-sucrose-glycerophosphate broth. All stained with safranin except (l), which was Giemsa stained: ×2000.

(a) and (b). Clover organism 144B, *Arthrobacter*: (a) 6 hr old, (b) 24 hr old.

(c) and (d). Clover organism 39, *Arthrobacter*: (c) 24 hr old, (d) 48 hr old.

(e). Rye-grass organism 278, *Arthrobacter*, 24 hr old.

(f). Rye-grass organism 176, *Arthrobacter*, 48 hr old.

(g) and (h). Rye-grass organism 311, *Arthrobacter*, 48 hr old.

(k). Clover organism 124, *Arthrobacter*, 48 hr old.

(l). Rye-grass organism 292, *Nocardia*, 72 hr old.

Plate 2. Cultures on sauerkraut-sucrose-glycerophosphate broth (a–d, g–l) or agar (e and f); (a)–(h), ×2000; (k) and (l), ×1600.

(a). Rye-grass organism 266, *Nocardia*, 24 hr old, safranin stain.

(b). Clover organism 50B, *Nocardia*, 48 hr old, safranin stain.

(c). Clover organism 18, *Nocardia*, 48 hr old, safranin stain.

(d). Clover organism 43B, *Mycoplana*, 48 hr old, safranin stain.

(e) and (f). Clover organism 54, *Mycoplana*: (e) 72 hr old, Giemsa stain; (f) 72 hr old, congo red cell wall stain.

(g) and (h). Rye-grass organism 254, *Mycoplana*, safranin stain: (g) 24 hr old; (h) 48 hr old.

(k) and (l). Clover organism 7, *Micromonospora*, Giemsa stain, 60 hr old.

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THE INFLUENCE OF ENVIRONMENTAL FACTORS ON THE MICROBICIDAL EFFECT OF IONISING RADIATIONS

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Contents

		page
1.	Introduction	96
2.	Microbial inactivation by radiation	97
3.	Factors modifying microbial inactivation	98
	(a) Number of organisms	98
	(b) Hydrogen ion concentration	98
	(c) Stage in the growth cycles of cultures, cells and spores	98
	(d) Degree of hydration	99
	(e) Temperature effects	99
	(f) Effect of the gas phase	101
	(g) Protective agents	103
	(h) Sensitizing agents	105
	(k) Effect of mineral imbalances	107
4.	Discussion	107
5.	Addendum. Doses required for the radiation processing of foods	108
6.	Conclusion	111
7.	Acknowledgement	111
8.	References	112

1. Introduction

If radiation sterilization is to come into use on a large scale in the food and pharmaceutical industries, a more exact knowledge will be necessary of the ways in which the microbicidal effect of ionising radiations can be modified. This paper sets out to summarize briefly the present state of knowledge of various physical and chemical modifying factors. To help fill the many gaps, and also possibly to suggest new lines of study, examples are included of relevant work with higher organisms. Work on these modifying factors has, besides its possible practical uses, great value as pure research, and results so gained are helping towards a better understanding of the primary and secondary effects of radiation on living matter. General aspects of the radiation sterilization of foods were reviewed by Hannan (1955), and microbiological aspects by Niven (1958).

2. Microbial Inactivation by Radiation

With metazoa, metaphyta, and most protozoa and algae, the effects of radiation can be observed by following the reactions of individual organisms. But bacteria, because of their small size, cannot be studied in this way and biochemical changes have to be demonstrated, in most cases, with relatively dense suspensions of organisms. The viability of bacteria is determined by their ability to form visible colonies on suitable nutrient media, but death is difficult to define. Failure to form a colony may imply the death of the original organism immediately or after a considerable delay, or death of the progeny of the original organism, it may be after many cell divisions. This is especially true of radiation killing, where bacterial suspensions may continue most of their metabolic processes (e.g. respiration, motility) for many hours after irradiation, although the suspension may be almost sterile as judged by the ability of the cells to form colonies. For this reason the term 'inactivation' is preferred to 'death' in such cases. The number of colonies formed is also markedly dependent upon the composition of the nutrient medium (see below).

Three types of survival curve have been described by Gunter & Kohn (1956*a*): exponential, sigmoidal and composite. The exponential curve, a straight line when the surviving fraction is plotted semilogarithmically against dose, is taken to indicate that each organism needs only 'one-hit' from the radiation to be inactivated. Examples of this type of survival curve are afforded by *Pseudomonas fluorescens* and haploid *Saccharomyces cerevisiae*. The composite curve (shown by *Escherichia coli* W–1484) consists of two such exponential curves intersecting. The population consists of sensitive and resistant cells which are genotypically homogeneous (Gunter & Kohn, 1956*a*). Sigmoidal survival curves are interpreted as indicating that each organism needs more than 'one-hit' to inactivate it for example *Azotobacter agile* and diploid *Sacch. cerevisiae*. If a population contains a very small proportion of highly resistant organisms, the curve may flatten out markedly at low survival levels to give a 'tail'. The nature of the sensitive molecules within the cells has yet to be established, but inactivation of these molecules appears to manifest itself as inhibition of specific syntheses, such as those of deoxypentosenucleic acid (DNA) or certain adaptive enzymes. If the sensitive molecule is DNA itself, the lesion would appear to be covered by the term lethal mutation. The question has been discussed by Kimball (1957).

The mode of action of ionising radiation is still unsettled. There are two schools of thought. The most widely held states that the primary ionisations occur mainly on water molecules and that the free radicals and ions so formed then attack chemically the organic part of the cell. On the other hand there is still a considerable body of opinion that direct ionisations in the organic molecules of the cell are responsible. It is surely possible that both types of action can occur and that their relative importance will vary according to the conditions of the experiment. However, convincing evidence to distinguish between direct and indirect actions of ionising radiations has not yet been secured with bacteria.

Some of the factors which may modify the effects of radiation are discussed below. It should be noted that the action of a given factor before or during irradiation may be different from the action of the same factor after irradiation, although no attempt has been made to subdivide the data on this basis.

3. Factors Modifying Microbial Inactivation

(a) *Number of organisms*

Over a wide range the fractional survival curve for a given organism is independent of the initial concentration of cells under controlled gas conditions. Koh, Morehouse & Chandler (1956) reported a protective effect as the concentration of their suspension of *E. coli* increased above a certain level, and a similar effect was reported for *Pseudomonas geniculata* (Doty *et al.* 1956). Gunter & Kohn (1956*b*) showed this to be due to removal of dissolved oxygen by the respiration of the dense suspension of vegetative bacteria. Most living organisms are considerably more resistant to radiation in the absence of oxygen. Bacterial spores are, however, much less affected by the presence of oxygen and this may explain why Pepper, Buffa & Chandler (1956) observed no protection of *Bacillus* spores when a large number of *E. coli* were present during irradiation.

In a commercial radiation sterilization process, the aim of normal working will be to keep the initial number of micro-organisms as low as possible. The possibility of a 'tail' of resistant organisms on the survival curve must always be borne in mind. This has not yet been reported with pure cultures of bacteria, but C. Kaplan (1958, to be published) has shown that it can apparently occur with viruses under certain conditions. It is of course most likely to occur in mixed cultures containing (for example) sporeformers.

(b) *Hydrogen ion concentration*

Earlier work (Moriarty, 1950) indicated that resistance to radiation was highest at neutral pH with *Staphylococcus aureus*, but later work on *Ps. geniculata* failed to show any difference (Doty *et al.* 1956). Little pH effect was found with spores of *B. subtilis* (Edwards, Peterson & Cummings, 1954) or F.S. 1518 (Pratt & Eklund, 1954), and Fuld *et al.* (1957) were unable to detect any difference with *B. subtilis*; but the resistance of *B. thermoacidurans* to β-rays and of *Clostridium sporogenes* to γ-rays varied with pH as follows: at pH 2·2 and 10·0 the resistance was the same, and less than that at pH 3·4. At pH 4·6 the resistance was larger still and equal to or less than that at pH 7·0, where it was highest.

The effect of the pH of the post-irradiation medium has been studied by Weatherwax (1956) and Alper & Gillies (1958*b*), who found that irradiated *E. coli* B survived better on acid than on alkaline media. Of interest here are the possibilities connected with recent suggestions for using adrenalin to raise the pH of meat and so reduce *post mortem* changes in conjunction with radiation (Zender *et al.* 1958).

(c) *Stage in the growth cycles of cultures, cells and spores*

Little comparative work has been published on this topic. Stapleton (1955) followed the variation in sensitivity to X-rays of *E. coli* B/r during the development of cultures. The percentage survival after 30,000 rads rose markedly during the lag phase but began to fall at the beginning of the logarithmic phase and reached its lowest value at the end of that phase. The percentage survival rose rapidly to its initial value when growth reached its stationary maximum. A maximum sensitivity in the logarithmic phase has also been reported by Spoerl *et al.* (1954). Houtermans (1953), using synchronized cultures of *E. coli*, showed that the sensitivity to α-particles

and ultraviolet radiation was greater just after division than in the preceding stages.

Spores have been reported to be more resistant than their corresponding vegetative forms (Koh, Morehouse & Chandler, 1956), but Laser (1956) found fresh *B. subtilis* spores to have about the same radiosensitivity as the vegetative forms. However, Laser's technique for measuring survival (manometric determinations of oxygen uptake) was sufficiently different from those generally in use for the results not to be strictly comparable. Proctor *et al.* (1958) found that spores have a tendency to lose their resistance when stored. It might be possible, by giving a mild heat treatment before irradiation, to get vegetative cells into the logarithmic phase. This could be of value in radiation food processing. A somewhat stronger heat treatment (say 60–80°) would appear to be necessary to inactivate enzymes in some foods, and this might also induce germination of some of the spores. Heat treatments are discussed in section (e).

(d) *Degree of hydration*

Moos (1952) and Lawton & Bellamy (1954) demonstrated that vegetative bacteria were more resistant when irradiated in a dry condition, and this was taken as evidence in support of the indirect action theory. Alper (1956) has, however, proposed an equally plausible explanation based on the direct action theory. The effect of water content is not a simple one, as has been demonstrated with barley seeds by Caldecott (1955, 1958), and there are two reports that bacterial spores are more sensitive in the dry state than when wet (Pepper, Buffa & Chandler, 1956; Houtermans, 1956). In the light of these divergencies it would seem wise to await further evidence before attempting to draw any conclusions as to the rôle of water in radiobiological damage. The effect of the removal of free water by freezing is discussed below.

(e) *Temperature effects*

Freezing might be expected to confer protection upon micro-organisms by preventing the mobility of free radicals and other reactive substances within the cell. A reduced sensitivity in the frozen state has, in fact, been demonstrated for *Staphylococcus aureus* 209 (Lawton & Bellamy, 1954) and *E. coli* (Hollaender & Stapleton, 1953; Fuld, Proctor & Goldblith, 1957), which were considerably protected by freezing. A similar effect was also shown for yeast by Wood (1954). Stapleton & Edington (1956) obtained results which were consistent with the hypothesis that sub-freezing temperatures protect not only by interference with the diffusion of a toxic agent to a sensitive site within the cell, but also by interference with the production of this toxic agent.

Bacterial spores are much less affected by freezing (Hannan, 1955, p. 57). Several reports indicate that they are more resistant in the non-frozen state (Pepper, Buffa & Chandler, 1956; Edwards, Peterson & Cummings, 1954). This was confirmed by Fuld, Proctor & Goldblith (1957) for *B. subtilis* but not for *B. thermoacidurans* or *Cl. sporogenes*, which showed no difference.

If it is accepted that the protective effect of freezing is not shown by spores, it might be inferred that for the protective effect to occur the cell must be in a certain physiological state, possibly a state dependent on active metabolism immediately preceding or immediately following freezing. Alternatively, it could be simply that

the spore contains its water in a bound form, so that there is no aqueous phase in which free radicals could travel. Sub-zero temperatures could not freeze a non-existent water phase, but could conceivably damage (e.g.) the spore wall in conjunction with radiation and thus cause a lowering of radiation resistance. Equally tenable explanations can be put forward on the basis of the direct action theory (Alper, 1956).

Spores also appear to differ from vegetative organisms with regard to the combined effects of heat and radiation. Morgan & Reed (1954) reported that bacterial spores were not sensitized by preheating but that irradiation sensitized them to post-heating. This has been confirmed for *Cl. botulinum* (Kempe, 1955; Kempe, Graikoski & Bonventre, 1957) and for *B. cereus* var. *terminalis* and Cameron's putrefactive anaerobe No. 3679 (Kan, Goldblith & Proctor, 1957). Kan and his colleagues concluded that irradiation sensitizes spores to subsequent heat by altering some protein constituent and thereby making it more heat sensitive. There has been somewhat less work on vegetative organisms but Langendorff, Langendorff & Sommermeyer (1953, 1954) reported *E. coli* to be sensitized to irradiation by preheating to 45°. Wood (1954) showed that *Sacch. cerevisiae* was sensitized by preheating to 52·5°, and also that holding at 52·5° during irradiation was more effective than heating to that temperature before or after irradiation. Powers, Webb & Ehret (1958) investigated the effects of temperature on the radiosensitivity of vacuum dried spores of *B. megaterium* from 5°K to 404°K and reported maximum sensitivity at 309°K, minimum sensitivity at 350°K, and a plateau of temperature independence below about 130°K.

Since bacterial spores are the most frequent radio-resistant organisms present in foods, one would expect their heat-irradiation characteristics to dominate the sterility requirements. This would appear to be so from the work of Erdman & Watts (1957) who, working on cured ground pork, found that preheated (to 70°) irradiated samples showed spoilage after 14 days whereas post-heated irradiated samples were unspoiled after 170 days. These spoilage tests did not involve bacteriological examinations, however. It should be noted that *Cl. botulinum* is among the most radiation-resistant organisms and that the problem of practical sterility revolves in large measure around the radiation resistance of that organism.

There is evidence that partial recovery from radiation damage can occur when certain bacteria are incubated at suboptimal temperatures after irradiation (Stapleton, Billen & Hollaender, 1953; Stapleton, 1955). Stapleton (1955) and Stapleton, Sbarra & Hollaender (1955) postulated that two processes followed the irradiation of *E. coli* B/r. One, having the same temperature coefficient as growth, was supposed to be synthetic in nature. The intervention of some destructive process with a higher temperature coefficient occurs at higher temperatures, and the overall survival at any temperature depends on the relative rates of these two processes. Evidence was cited that several systems are implicated in the *E. coli* B/r post-irradiation synthetic process, involving amino acid, purine and pyrimidine metabolism. Alper & Gillies (1958*b*) were, however, unable to demonstrate suboptimal temperature recovery with *E. coli* B; but this was because they did not use sufficiently low temperatures (Pratt, Moos & Eden, 1955). Jacobson (1957) gave evidence that recovery from X-ray

damage to the alga *Chlamydomonas reinhardi* was blocked at sub-optimal temperatures. From a technological aspect irradiation at non-ambient temperatures is in some instances of advantage in reducing the production of off-flavours; for example, off-flavour production in raw beef is minimized if this is irradiated in the frozen state (Hannan, 1955). In such cases it is obviously imperative to study the microbicidal effects of irradiation under the appropriate conditions. Low temperature irradiation has the advantage that the numbers of organisms will be kept down, while elevated temperatures may cause germination of spores. Either could therefore lower the dose required but would increase the overall cost of processing, and irradiation at other than ambient temperatures is therefore to be avoided unless of definite value.

(f) *Effect of the gas phase*

It has been known for some time that the presence of oxygen increases the lethal effect of radiation (Anderson & Turkowitz, 1941; Thoday & Read, 1947, 1949; Giles & Riley, 1949; Giles & Beatty, 1950). Hollaender, Stapleton & Martin (1951) showed that a washed suspension of *E. coli* in phosphate buffer was three times more sensitive to X-rays in oxygen than in nitrogen. Fuld, Proctor & Goldblith (1957) compared the sensitivity of various bacteria under different atmospheric conditions and their results are summarized in Table 1. Alper & Howard-Flanders (1956), Alper (1956) and Howard-Flanders & Alper (1957) studied the effect of irradiating at different partial pressures of oxygen on the radiosensitivity of *E. coli* B and other bacteria.

Table 1. *The effect of oxygen on the sensitivity of different bacteria to irradiation with* β- *and* γ-*rays*

(Data from Fuld, Proctor & Goldblith, 1957)

Organism	Relative sensitivities in O_2, air, vacuum and N_2
B. thermoacidurans	O_2=air=N_2=vacuum
E. coli	O_2>air>vacuum=N_2
B. subtilis	O_2>air>vacuum>N_2
Cl. sporogenes	O_2>air>N_2

Oxygen was effective at very low concentrations; with *Shigella flexneri*, 0·01 atmospheres partial pressure of oxygen was sufficient to confer 95% of the oxygen-dependent sensitivity, and for several strains, the maximum sensitivity was attained at concentrations corresponding to 2% partial pressure of oxygen in the gas phase. Hannan (1955) suggested that the oxygen effect could be explained on the basis of the indirect action theory, the effect of dissolved oxygen being to increase the number of .OH or .HO_2 radicals, or peroxides formed by the radiation, but he stressed that metabolic changes may be involved. The fact that bacterial spores show a much smaller oxygen effect than vegetative cells argues in favour of this. Alper (1956) discussed possible mechanisms and gave evidence for eliminating the peroxide hypothesis and the free radical hypothesis, except in very dilute solutions with little organic matter. She concluded that the initial lesion is caused by direct action of the radiation on the organism and that oxygen may then act on the initial lesion to

cause complete inactivation. Laser (1954) reported that metabolic inhibitors can simulate oxygen removal for some micro-organisms and suggested that the radiosensitivity depends on the state of the respiratory enzymes. Alper & Howard-Flanders (1956) have criticized this view on the ground that the respiration rate of bacteria reaches a maximum at considerably lower oxygen concentrations than does the radiosensitivity, and Graevskĭ & Konstantinova (1957) were unable to demonstrate the effect with *E. coli* using KCN or CO. Laser has developed his hypothesis further in a recent symposium (Laser, 1956). Kimball & Gaither (1953) showed that *Paramecium* exhibits no oxygen effect for vegetative death and division delay when catalase is present in the medium, but does in its absence. This would indicate that hydrogen peroxide formed from the water is responsible for the oxygen effect in these instances. Lethal mutations, however, show an oxygen effect even in the presence of catalase, so that a different mechanism must exist. Other results with protozoa are discussed by Kimball (1957).

A completely different effect is observed with coliphage T_1, where oxygen *protects* against radiation inactivation (Bachofer & Pottinger, 1956). These workers concluded that the protective effect of oxygen is due to a reaction between phage and oxygen. The resulting complex is assumed to confer stability on the phage. There is now, however, ample evidence that viruses are exceptions to the generally observed oxygen effect. *Shigella flexneri*, given nitric oxide in place of oxygen in the atmosphere, shows the radiosensitivity normally associated with aerobic conditions, even though all aerobic metabolism has been halted (Howard-Flanders, 1957). Oxygen is a biradical and nitric oxide a stable free radical, and the author suggests that the primary lesion of ionising radiation is the formation of a radical on a carbon atom belonging to a molecule essential to survival. If this radical combines with another radical (e.g. oxygen, nitric oxide) then the lesion becomes lethal. Ebert, Hornsey & Howard (1958), using similar experimental conditions, showed that if sufficient inert gas is added to the air in which *Sh. flexneri* is irradiated, then the sensitivity decreases to the anoxic level. Argon was more efficient than nitrogen, but neither gas produced any effect in the absence of oxygen. The authors concluded that the inert gases at high concentration can displace oxygen from sites within the cell at which oxygen molecules can increase the lethal effect of radiation. A lipid-water interface is mentioned as being a possible site.

Other recent reports of interest in this connection are Fetner's observations (Fetner, 1958) that chromosome breakage in *Vicia faba* by ozone and X-rays is additive, and the claim of Fenn *et al.* (1957) that a high tension of oxygen has a mutagenic effect on *E. coli* comparable with that of irradiation.

The composition of the gas phase can within limits be readily and cheaply modified on a commercial scale. Complete oxygen removal may be difficult, but with meats and other respiring tissues a delay between packing in a sealed container and irradiating will allow the partial pressure of oxygen to fall. The effect of the irradiation on the food itself may be modified by altering the gas phase. Thus cured meats readily lose their colour when irradiated in air but retain it in vacuum. This sort of effect may be so marked as to permit one type of gas packing only, irrespective of bactericidal effect.

(g) *Protective agents*

A distinction should be made between chemicals which exert their protective effect only when added before or during irradiation, and those which assist the cell to recover when added after irradiation. In the first class there are at least three types of effect. Hannan (1955) stated that "—SH compounds such as cysteine are examples of those which probably act as acceptors of free radicals. A second group such as alcohols, nitrite and formate may act by modifying metabolic processes, while others, such as sodium hydrosulphite, may simply combine with free oxygen to create anaerobic conditions". Since these words were written, however, it has become increasingly clear that most protective agents act, at least in part, by interference with the oxygen effect, and that the action of —SH compounds in particular is much more complex than the statement above would suggest. It has been established that not all —SH groups can be regarded as radiation protection agents in mammals (Koch, 1957*a*). Langendorff & Koch (1954, 1956) concluded that a special chemical configuration is required of the general formula

$$HS \text{——} (CH_2)_x \text{——} N \begin{matrix} \nearrow R_1 \\ \searrow R_2 \end{matrix}$$

(where R_1, R_2 are alkyl groups and x is not greater than 3). Similar conclusions have been reached by Doherty, Burnett & Shapira (1957) and Hollaender & Stapleton (1956), although both sets of workers stressed that greater protection was obtained with the amino group unsubstituted. Besides the simple free radical acceptor theory mentioned above, it has been suggested that these compounds act by the temporary masking of vital protein groups (Eldjarn & Pihl, 1955) or by ". . . direct reaction with radiation-induced radicals to form resonance-stabilized hybrids that react preferentially with other radicals and not with sensitive biological material." (Doherty, Burnett & Shapira, 1957). Compounds of this cysteamine group are very efficient protective agents for bacteria (Hollaender & Doudney, 1954; Hollaender & Stapleton, 1955, 1956), this being due, at least in part, to production of anoxia within the cell, and cysteamine itself is more effective with bacteria than with mammals. The effectiveness of cysteamine has been found to vary according to the strain of *E. coli* used (Hollaender *et al.* 1958). Another group of protective agents, related to S-β-aminoethyl*iso*thiouronium bromide HBr, was less effective with bacteria. Significant protection of *E. coli* by cysteamine could still be obtained if the cells were washed immediately before irradiation, a large part of the compound presumably being retained within the cell. Bridges, Olivo & Chandler (1956) were unable to show this effect with cysteine HCl on two species of *Candida*. Fuld, Proctor & Goldblith (1957) tested the protective effect of 1% sodium ascorbate on various organisms, and demonstrated a dependence on the type of radiation and on the suspending medium. For β-radiation, *B. subtilis* was protected by ascorbate in pea purée and saline and *B. thermoacidurans* and *Cl. sporogenes* in pea purée but not in saline. For γ-radiation, *Cl. sporogenes* was protected by ascorbate in both pea purée and saline. But not all bacteria are protected by these chemicals: *Ps. geniculata* survived equally after γ-radiation whether ascorbic acid, nitrite, glutathione, cysteine or thioglycollate were present or not (Doty *et al.* 1956), though an unidentified substance in meat juices

did increase the radioresistance of this organism. In this connection it must be realized that any metabolite which stimulates respiration will tend to deplete the medium of oxygen and may thus act as a protective agent. For this reason the higher the organic content of any medium, the higher, generally, will its protective power be. This was found to be true by Fuld, Proctor & Goldblith (1957) for *E. coli*, A. *thermoacidurans* and *Cl. sporogenes* but not for A. *subtilis*, which was more sensitive in pea purée and milk than in saline. Sokurova (1956) reported, however, that two species of *Azotobacter* were more sensitive when irradiated in a sugar-mineral medium than when irradiated in water, and believed this to be connected with the metabolic state of the cells at the time of irradiation, and Laser (1954, 1956) obtained a protective effect, which simulates the removal of oxygen, by means of metabolic inhibitors.

It is known that various environmental conditions after irradiation can alter the effects of the radiation. If after such treatments the survival is greater than in the 'normal' control, then 'recovery' is said to have occurred. Metabolites added after irradiation have been found to promote the 'recovery' of *E. coli* B/r (Stapleton, Sbarra & Hollaender, 1955). Spleen extract was most active and some progress has been made towards isolating the factors involved. Much of the activity appeared to be in four amino acids, glutamic acid, serine, methionine and tryptophan, plus guanine and uracil. Woese (1958) found a similar effect with vegetative cells of *Bacillus* species but detected little or no difference with the corresponding spores on minimal or complete media. Donellan & Morowitz (1957) observed a similar lack of post-irradiation effect with *B. subtilis* spores. The mechanism of the recovery of *E. coli* B/r has been discussed by Hollaender & Stapleton (1956), who interpreted their data as indicating that much of the 'recovery' involves an adaptive process, possibly new enzyme synthesis, which has certain nutritional requirements. This interpretation has been criticized by Alper & Gillies (1958*b*), who questioned the validity of these conclusions from the published data. Spores of *Streptomyces* were found to exhibit a complex response to various treatments after X-irradiation (Wainwright & Neville, 1955).

The addition of magnesium ions to *Hydra* after X-irradiation has a beneficial effect on survival (Daniel & Park, 1954; Park, 1956). After 4,500 r of X-rays the budding of *Hydra* was significantly inhibited, but the post-irradiation addition of $MgSO_4$ did not affect this. Post-irradiation addition of $MgSO_4$ together with glutathione, however, gave complete protection (i.e. normal budding) over a fairly wide range of concentrations. Glutathione alone partially reversed the inhibitory effect of the radiation on budding. Mention should be made here of one substance which was observed to have a protective effect on mammals when in fact sensitization had been expected, namely cholestenone hydroperoxide (see below).

The use of chemical additives in food is not widely favoured. However, provided it can be shown that they are not harmful their use is being permitted. Compounds designed to protect the flavour of food from the effect of radiation are being studied and their effect on bacterial radiosensitivity must be considered. An ideal substance would of course confer flavour protection while increasing bacterial sensitivity. Prolonged feeding tests would be required before the commercial use of any such compounds would be permitted.

(h) *Sensitizing agents*

The search for radiation protecting agents is being conducted on a rational basis. It is difficult to find any such basis for the search for sensitizing agents. There are a number of reports of such agents, both for higher organisms and (to a lesser extent) for micro-organisms, but with few exceptions they seem to have been discovered after very slender theoretical deduction and are as yet unconfirmed. A sensitizing agent should be regarded as a substance which enhances the effect of radiation without itself being toxic; or if it is toxic, the resulting effect should be more than the sum of the radiation effect and the agent's effect separately.

The earliest reports came from tumour therapy research. Mitchell & Simon-Reus (1947) and Mitchell (1954) reported a striking enhancement of the action of X-rays on tumours by Synkavit (tetrasodium 2-methyl-1:4-napthohydroquinonediphosphate) and similar synthetic vitamin K substitutes. No significant sensitizing effect was found when Synkavit was present during irradiation of suspensions of *E. coli* or *Sacch. cerevisiae* (Kohn & Gunter, 1955), when present during growth and irradiation of *E. coli* B/r (Howard-Flanders & Alper, 1957), or when it was present in the post irradiation plating medium (Bridges, B. A., unpublished observations). Hanel, Hjort & Purser (1958) have recently shown Synkavit to be radiomimetic in effect on cholesterol synthesis in mice and to cause nuclear aberrations in some mouse liver cells. The combined effect of Synkavit and X-radiation was, however, less than the effect of either treatment alone. Carpender & Lanier (1950) observed enhancement of X-ray damage to tumours with 8-azaguanine. This substance did not affect the radiosensitivity of *E. coli* B/r suspended in quarter-strength Ringer's solution or phosphate buffer when it was present at 10^{-3} M for varying periods before irradiation with 30,000 rad of Co^{60} γ-rays, and when present in the plating medium at 10^{-3} M made no difference to the counts of *E. coli* B/r either before or after irradiation (Bridges, B. A., unpublished observations). Peters, Gartner & Krais (1955) reported that Megaphen (N—(3′-dimethylamino) propyl-3′-chlorophenothiazine) and γ-radiation had a synergistic action on Ehrlich ascites tumour cells.

Woollam, Pratt & Fozzard (1957) found a fourfold increase in radiation induced damage (anophthalmia) of the embryo when pregnant rats were fed with excess vitamins A, B, C, D and E. In experiments with rats and mice, thiamine pyrophosphate (vitamin B_1) and riboflavin (vitamin B_2) were found to be true sensitizing agents (Langendorff, Koch & Hagen, 1956). Koch (1957*a*) reported the derivation of some sensitizing agents (using rats and mice as assay organisms) from the cysteine-cysteamine group of protective agents. These molecules may be chemically altered, for example by moving the $-NH_2$ group to the β-position or by replacing it by an alcoholic —OH group. By such means β-homocysteine, isocysteine, thioglycol, and α-methyl cysteine were made and found to be sensitizing agents. Koch (1957*a*) suggested that the cysteine-cysteamine group of compounds should be regarded as metabolites and the corresponding sensitizing bodies as antimetabolites. From the ideas of Eldjarn & Pihl (1955), the protective bodies are regarded as masking vital endogenous —SH groups during irradiation, and Koch suggested that the sensitizing agents also mask but persist sufficiently long for toxicity to occur. He concluded "It is obvious that they have the same affinity for definite endogeneous —SH groups

as have the protective bodies". One criticism of this theory is that any masking substance which persists long enough to cause toxicity should surely be toxic irrespective of whether irradiation occurs during this period and should not therefore be classified as a true sensitizing agent. In the view of the present authors it seems more probable that the complex formed between protective –SH bodies and vital enzyme –SH sites is inherently more stable, and that formed by sensitizing –SH bodies inherently less stable, than the natural states of the vital –SH sites towards ionising radiation. Another possibility is that the so-called 'sensitizing agent' is in fact converted by radiation into another compound which is the actual toxic masking agent. A new approach was recently reported from the Massachusetts Institute of Technology. A way was being sought of inducing radiation resistant bacterial spores to germinate into the much less resistant vegetative forms, and α-alanine and adenosine were found to have this effect when added to suspensions of *B. cereus* spores in (presumably) water. In fact the radiosensitization was far in excess of the heat sensitization (Kan, Goldblith & Proctor, 1958). α-Alanine and α-alkyl alanines have also been found to sensitize laboratory mammals to radiation (Langendorff & Koch, 1955). In experiments with the yeast *Sacch. ellipsoideus*, Kiga, Ando & Koike (1955) reported a radiosensitizing action of malonic acid at 10^{-3} M which was most apparent at the lower dose levels (10^4–2×10^4 r). Malonic acid is known to inhibit oxidative phosphorylation and, very specifically, succinic dehydrogenase. The addition of fumaric or aspartic acid to the post-irradiation medium completely nullified the effects of the malonate, presumably by overcoming the biochemical blockage produced by malonate in the Krebs cycle. Cysteine afforded partial protection when added after irradiation. Maleic acid, an inhibitor of –SH enzymes, possessed a sensitizing effect similar to that of malonic acid. Among the sensitizing agents reported by Koch are Syntobiase and methylandrostendiol (Koch, 1957*a*), methylene blue, fluorescein (Koch, 1957*b*), testosterone, dehydroandrosterone, and cholesterol (Koch, unpublished). As these substances suggested a link between carcinogenicity and sensitizing properties, tests were made (Koch, 1957*b*) on rats with 3:4-benzpyrene (a carcinogen) and 1:2-benzpyrene (no carcinogenic activity). The former was found to be a sensitizing agent and the latter was inactive. Δ 6-cholestenone –3-β –ol-5-α-hydroperoxide, which was expected to have a sensitizing effect, did in fact offer significant protection (Koch, 1958).

Alper & Gillies (1958*a*,*b*) reported that an unidentified factor present in peptone had an inhibitory effect on *E. coli* B (and some other bacteria) after irradiation. Since this factor is present at a higher concentration in some brands of media (e.g. Oxoid) than in others (e.g. Difco), the authors suggested that this may explain discrepancies observed between results from different laboratories. These observations are also of interest in the light of the results of Jacobs & Harris (1953) who noted substances in peptone and meat extracts which affect bacteria damaged by phenolic antiseptics more than they affect normal bacteria. The synergistic action of ethylenediaminetetra-acetate (EDTA) is discussed below.

No antibiotics have yet been shown to be sensitizers for radiation, but work is being carried out concerning the use of antibiotics in conjunction with radiation since their effect on the survivors of mixed populations may be favourable for the preserva-

tion of food. Niven & Chesbro (1956) have reported their results on the complementary action of antibiotics and irradiation in the preservation of fresh meat, and Liston & Shewan (1957) briefly described the effect of γ-radiation on cod treated with chlortetracycline.

The general remarks made in the preceding section concerning the commercial use of protective agents apply also to sensitizing agents. The link between agents which sensitize mammals to radiation and their possible carcinogenicity must also be noted in this respect.

(k) *Effect of mineral imbalances*

Steffensen (1953, 1955) found that there were more spontaneous chromosome aberrations in *Tradescantia* grown with suboptimal magnesium and calcium than in normal plants. Subsequently calcium deficiency (but not magnesium deficiency) was also found to increase the rate of radiation induced chromosome aberrations (Steffensen, 1956, 1957). The radiosensitivity of *Aerobacter aerogenes* was not affected by growing it in media deficient in magnesium but a *Pseudomonas* sp. was slightly more sensitive to γ-radiation when grown in magnesium deficient media (Bridges, B. A., unpublished observations). Jacobson (1957) found no difference between normal cells of *Chlamydomonas reinhardi* and cells deficient in magnesium and calcium. Bair & Hungate (1958) reported a synergistic effect on *Sacch. cerevisiae* between EDTA and irradiation, the source of radiation being dissolved plutonium or tritium. There was no synergistic effect when EDTA was added before or after a single dose of 300,000 r of X-rays and a general change in the electrolyte balance was believed to be responsible for the effect, rather than a specific calcium deficiency, since the effect of the EDTA was not nullified by the addition of calcium.

The effect of various inorganic ions on *Micrococcus pyogenes* var. *aureus* has been investigated by Dunn (1953). He concluded that at 0·5% definite enhancement of germicidal action occurred with cadmium and with cobalt, possibly slight enhancement with calcium and nickel, and some protection with lithium and ferric ions (all as chlorides). At 5%, cobalt chloride, aluminium sulphate and nickel chloride enhanced the lethal effect, lithium and calcium chlorides impaired it, and potassium sulphate had no appreciable influence. Ferrous ions afforded considerable protection for *Ps. geniculata* if they were autoclaved with glucose or tryptone prior to irradiation and if citrate was used as a solubilizing agent (Doty *et al.* 1956). The product of the glucose-ferrous salt reaction has not yet been identified.

As discussed above, the addition of magnesium ions to *Hydra* after X-irradiation was found to be protective by Daniel & Park (1954) and Park (1956).

The differences in radiosensitivity due to mineral imbalances are usually small and they would appear to be unimportant in food technology.

4. Discussion

There are two ways in which knowledge of the experimental modification of radiation damage is of direct relevance to radiation sterilization. First, without this knowledge small alterations in environmental conditions could conceivably be permitted unintentionally to lessen the lethal effect of the radiation and allow one or two organisms

to survive. Second, since the dose necessary for effective sterilization (in excess of 2 megarad) also causes deleterious odour, flavour and texture changes in many foods, a study must be made of the conditions under which the lethal effect is greatest so that sterility may be obtained with the lowest possible (and therefore cheapest) dose. Many of the factors discussed in the preceding sections are controllable only with difficulty in foods, and there remain three with which a successful result might be obtained; temperature changes, composition of the gas phase, and chemical additives (including antibiotics).

Numerous combinations of temperatures before, during and after irradiation are possible, and many have yet to be tried. The reversibility of a part of the radiation damage to bacteria has been described and it would appear to be necessary to prevent post-irradiation recovery in foods. Careful control of the temperature for the 12 hr immediately following irradiation seems to be the best way of achieving this. Control of the gas phase is feasible but more work is needed before a specific treatment can be proposed. Little thought was given, until recently, to the possibility of using chemical additives in combination with radiation, either as sensitizing agents, spore germination inducers, or bacteriostatic agents in their own right. The addition of chemicals to food destined for human consumption is strictly controlled by law in most countries, and the use of antibiotics is liable to bring forth objections from the medical profession and from workers in the field of chemotherapy who are already much troubled by the increasing incidence of drug-resistant strains (Knox, 1958, p. 304).

Although spores are the most frequently encountered resistant micro-organisms, sensitive vegetative cells will be present in foods in much larger numbers. Therefore vegetative cell death is of more interest in 'pasteurization' procedures designed merely for extension of shelf life, and spore death is more important when dealing with sterilization proper. But conditions which affect vegetative cells do not necessarily affect spores in the same way. Consequently conditions which are optimal for 'pasteurization' may not be so for sterilization, and *vice versa*. There will also be certain types of food in which radiation-resistant micrococci are likely to be troublesome (Anderson, Nordan & Cain, 1956). In considering, therefore, the use of such modifying factors, each food or pharmaceutical must be considered separately with reference to the type of preservation required.

We would conclude by emphasizing how much more work is needed on the microbiological side before one can be reasonably confident about the safety and economics of any suggested radiation preservation process.

5. Addendum. Doses Required for the Radiation Processing of Foods

In the above discussion, some of the factors which could conceivably modify the doses necessary to process foods have been described. There is, however, a certain amount of confusion among those not intimately connected with the field, as to what these doses signify, and how they are derived, and it was therefore considered that this explanatory addendum should be provided.

'Inactivation' doses have been published for many micro-organisms. The usual method of determination is to take half a dozen tubes containing a fairly dense suspension of the organism in question, give them different doses of radiation, and then use ordinary plating techniques to discover which dose was sufficient to prevent any organisms being detected. 'Sterilizing' doses for many foods have been determined in a similar way. In both instances the figures are of limited value, since although the initial concentration of cells may be known with some accuracy, there is no knowledge of the final concentration.

High dose procedure

'Sterilizing' doses will depend on several variables, including the standard required, the environmental factors, and the numbers and types in the initial flora. Hannan (1955) suggested that a standard of 10^{-8} viable organisms/g is commonly achieved with heat processed foods, and there is no reason why the standard should be lower for radiation processed foods. Environmental factors have been dealt with above. As regards the initial flora, if a standard of 10^{-8} surviving organisms/g is required the inactivation factor will lie between 10^8 and about 10^{14} or more, according to the initial degree of contamination. With normal techniques one cannot investigate inactivation factors much greater than 10^7, and so with pure cultures one must extrapolate the survival curve to the limit required. There is no evidence that this introduces any great errors when pure cultures of micro-organisms are involved, but with mixed cultures, such as are found in foods, the semilogarithmic survival curve does not approach a straight line and therefore cannot be extrapolated. Proctor & Goldblith (1952) suggested that the commercial 'sterilizing' dose may be obtained by extrapolating the survival curve for the most resistant organism found in the food, starting at the most probable concentration of that organism. This should be a good approximation, since the rate of inactivation of a given species is independent of the presence of other species. However, if a less resistant organism is present in excessively large numbers, some may survive after the smaller numbers of resistant organisms have been inactivated, although this is unlikely to occur in practice.

Clostridium botulinum is generally regarded as among the most resistant organisms to be found in food, leaving aside the highly resistant micrococci whose presence has been reported in certain meat products. In Fig. 1 a typical survival curve has been extrapolated down to 10^{-8} organisms/g. This curve is based on data obtained with *Cl. botulinum* by Morgan & Reed (1954), but it will be assumed that it applies to some unspecified sporeformer. It can be seen that for a food with only 1 10 such spores/g, the sterilizing dose (as defined above) will be about 3 Mrad. For a food containing 10^3 resistant spores/g, the dose would be 4 Mrad, and the dose of 4·8 Mrad, sometimes quoted as the required sterilizing dose for all foods, will reduce a total population of 10^5 spores/g ,with a radiation resistance comparable with that of *Cl. botulinum*, to 10^{-8}/g. As it is unlikely that the population of *Cl. botulinum* spores in any food will exceed 10^1–10^2/g, the numbers left after a dose of 4·8 Mrad will therefore be 10^{-11}–10^{-12}/g.

Such low values will not be achieved if the initial numbers present are greater than those given above, or if the irradiation resistance is greater than that indicated

in Fig. 1. This might occur through the action of some protective agent in a particular foodstuff, and 'sterilizing' doses will therefore have to be determined for each food individually.

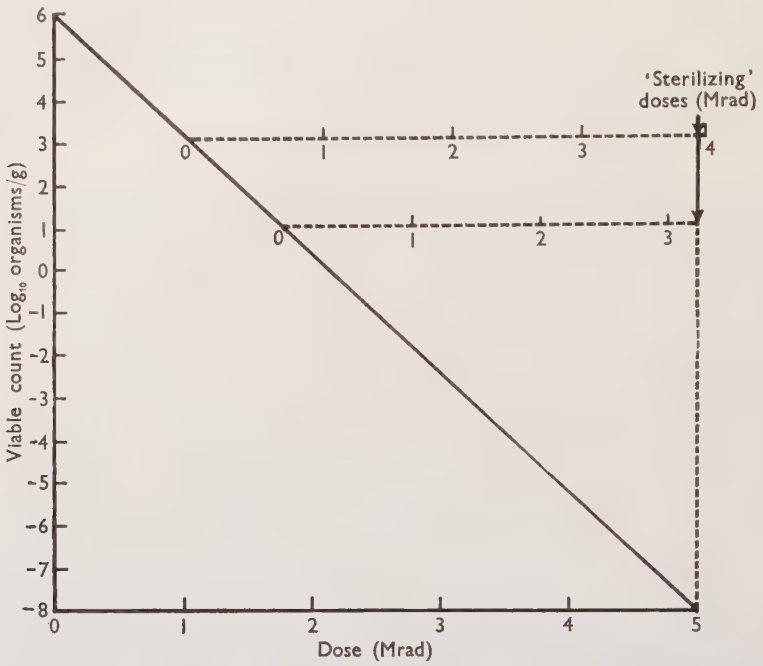

Fig. 1. The destruction of radio-resistant spores by γ-rays, extrapolated from the data of Morgan & Read (1954) on *Cl. botulinum*, with the 'sterilizing' doses required at two initial population levels to achieve a final standard of 10^{-8} organisms/g.

As with other forms of processing, a great reduction in sterilizing dose would be obtained if a reliable method could be devised of inducing the germination of bacterial spores beforehand. This might be done either with a short incubation or by chemical means. Kan, Goldblith & Proctor (1958) have published some promising results in which pre-irradiation germination was effected by alanine or adenosine, and in which the sensitization to radiation was greater than to heat; but there is no method yet available which will certainly give 100% germination.

Low dose procedure

Two main types of process are involved here. First, there is the complete eradication of a particular pathogen, an example being the destruction of salmonellas in whole egg pulp. The dose necessary may be calculated by extrapolation as before. Second, there is the procedure of irradiation with 5×10^4–5×10^5 rad, aiming not at sterilization but at greatly lowering the microbial count and thereby extending the shelf life of the product. This process is frequently termed 'radiopasteurization'. What may be a serious drawback to this procedure is that the residual microbial flora is different

from the normal, and spoilage, when it occurs, may be of a different type from normal, perhaps a type not easily recognizable by the consumer. It has been estimated (Hannan, 1955) that in general a dose of 10^5 rad would reduce the microbial population of a food product by a factor of 10^2 or 10^3, and that this might be expected to give an increased storage life with many foods, especially at refrigeration temperatures.

Low or moderate doses may also be used in conjunction with other factors such as acid conditions, low temperature storage, and mild heat treatment (Ingram, 1958).

One point which is not often mentioned and which we feel should not be overlooked concerns the state of the micro-organisms which survive a radiation process. It is well known that those which survive a commercial heat process are frequently in a dormant state and may remain so for long periods, especially in canned foods, although they are viable when transferred to other growth media. It is not known whether bacteria which survive irradiation may be kept in a similar dormant state. It is a common experience among workers in this field that radiation survivors may need 2–3 weeks incubation in broth before growth occurs, and the work of Stapleton (1955) and others has shown that certain bacteria may recover from the effects of irradiation if they are incubated at suboptimal temperatures or in the presence of certain metabolites. It would therefore seem desirable that further work should be done on this topic.

6. Conclusion

Having reviewed the evidence, the present writers conclude that while the use of low doses of radiation, either as pasteurization procedures or in combination with other treatments, shows some promise (see Ingram, 1958), there is little justification for optimism about the application of radiation 'sterilization'. Radiation damage to flavour, etc., is extensive at the doses which are necessary for commercial 'sterilization' and there is no likelihood that more than a very few products so treated will gain public acceptance.

A solution to the problem could be approached in two ways. First, to discover means of reducing these changes, and second, to discover means of so sensitizing the micro-organisms that sterilization can be achieved with a much lower dose of radiation. Some limited success has been achieved by the first approach. It is now the responsibility of the microbiologist to investigate the second. The whole outlook for radiation processing of foods would be radically changed if, for example, 'sterilization' could be achieved with 5×10^5 rad, and the range of radiation resistance over all species is so great that one can perhaps hope for some progress in this direction.

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OBSERVATIONS ON THE ISOLATION OF SALMONELLAE FROM SELENITE BROTH

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SUMMARY: Studies of growth curves of enterobacteria in selenite broth containing different carbohydrates showed that whereas mannitol and lactose brought about a steep fall in coli-aerogenes bacteria, mannitol improved the growth of *Salm. typhi-murium*. With mixed cultures of *Cit. freundii* I and *Salm. typhi-murium* the presence of lactose, utilizable by the former, adversely affected the viable count of the latter.

Comparative studies with routine faeces specimens showed that selenite broth was an efficient selective medium with MacConkey's agar; but much better results were obtained when it was combined with deoxycholate-citrate agar. Gentian violet introduced into selenite broth improved its selectivity for most *Salmonella* types when MacConkey's agar was used for final isolation.

Selenite broth is probably the most widely used enrichment medium for the isolation of salmonellae. Though comparative studies have shown it to be highly efficient, nevertheless the failures that sometimes occur must give cause for concern. Occasionally, selenite broth inoculated with substantial quantities of food suspected of causing food poisoning has failed to yield salmonellae whereas minute inocula have shown it to be grossly contaminated with those organisms. Similar difficulties were encountered by Thomson (1954) with faeces of enteric carriers. Although the failure of one selective medium may be offset by using a variety of different media it seemed likely that modifications of selenite broth might achieve as much if more were known about the properties of the medium and the conditions which govern selectivity.

Leifson (1936) used lactose in his selenite medium, but remarked that other carbohydrates may be substituted, though seldom with any advantage. He found, also, that satisfactory results could be obtained without any carbohydrate. Hobbs & Allison (1945) advocated the use of mannitol but Williams Smith (1952), who compared selenite broth with lactose and with mannitol, found little difference between the two media. The question of the carbohydrate has not been satisfactorily settled and has been studied, therefore, particularly in relation to competition in mixed populations, which may adversely affect the isolation of salmonellae from contaminated materials.

Triphenylmethane dyes have frequently been used in selective media with the object of making them still more selective, but with variable results. In so far as selective media for salmonellae are concerned, most of the work has been done with tetrathionate broth and brilliant green. However, Stokes & Osborne (1955) used brilliant green in selenite broth to inhibit *Escherichia* and *Proteus*, but Osborne & Stokes (1955) found it was not entirely satisfactory for use with egg products. Some

results of experiments with gentian violet in selenite broth are also included in the present paper.

Materials and Methods

Media. Selenite broth was prepared according to Hobbs & Allison's modification (1945) of Leifson's formula (1936), in which mannitol is substituted for lactose and instead of two phosphate salts only the disodium salt is used, the pH being adjusted to 7·1 by means of N HCl. The medium was sterilized by steaming for 30 min. This selenite broth has been used throughout except when different carbohydrates have been introduced in comparative experiments. Each batch of selenite broth was usually prepared in bulk in 2 l. quantities and kept at 4°. No deterioration was noticed even after storage for 3 or 4 months. For use, 10 ml quantities were pipetted into carefully washed sterile screw-cap containers. Proteose-Peptone (Difco) was used in the growth curve studies, while another make of peptone in current use in the laboratory was satisfactory for faeces examination.

Mannitol-peptone broth was prepared in the same way as the selenite broth except that the sodium selenite was omitted and sterilization was carried out by autoclaving at 115° for 10 min.

An indicator medium was used for viable counts. It consisted of 2% agar, 2% peptone, and 1% of lactose or of sucrose (all as % w/v). Neutral red was added as in MacConkey's agar.

Inoculation and counting. The organisms used were isolated from routine laboratory specimens and included serotypes of *Salmonella*, *Escherichia coli* I, *Citrobacter freundii* I, *Klebsiella aerogenes* I, and *Proteus vulgaris*. The inoculum for the growth curve determinations was 0·02 ml of a 1:10 dilution of an overnight broth culture to 10 ml of selenite broth. Viable counts were estimated by the method of Miles & Misra (1938), using nutrient agar for cultures containing one species of organism and the indicator solid medium for cultures containing mixed organisms.

Results

The selectivity of selenite broth with routine faeces specimens

Although there is abundant evidence to show the value of selenite broth (SB) for the isolation of salmonellae, nevertheless in most instances comparisons have been made between different selective fluid media by plating out on selective solid media. The combination of two selective media, however, to some extent obscures the level of efficiency on both media. To obtain a clearer picture of the efficiency of selenite broth by itself, faeces specimens were examined by means of different combinations of selective and non-selective media. Direct platings were made on deoxycholate-citrate agar (DCA), and a loopful of faeces was inoculated into selenite broth and another into mannitol-peptone broth (MPB). After overnight incubation at 37° one loopful from each of the two fluid media was stroked out on MacConkey's agar (MA) and another on DCA, and the culture plates examined the following day.

The results for 139 faeces specimens positive for salmonellae are shown in Table 1. The number found positive by direct plating on DCA (68%) was low compared with

Table 1. *A comparison of direct plating on deoxycholate agar (DCA) with enrichment in selenite broth (SB) or mannitol-peptone broth (MPB) followed by streaking on MacConkey's agar (MA) or DCA, for the isolation of salmonellae from faeces*

Salmonella found	No. of positive samples	Numbers of faeces samples positive by				
		DCA direct	SB, MA	SB, DCA	MPB, MA	MPB, DCA
Salm. typhi-murium	62	44	58	62	15	56
Salm. paratyphi B.	37	24	35	34	3	27
Salm. bareilly	20	14	15	17	7	18
Salm. typhi	10	8	9	10	2	10
Salm. stanley	6	1	5	5	—	4
Salm. dublin	2	1	2	2	1	1
Salm. enteritidis	2	2	2	2	2	2
Totals	139	94	126	132	30	118
Percentages	100	68	91	95	22	85

those from either fluid medium with DCA as the solid medium. The combination of MPB and DCA yielded 85% of positive isolations, whereas selenite broth and DCA gave 95%. Selenite broth and MacConkey's agar gave 91% positive isolations. However, with MPB and MacConkey's agar only 22% were positive. As judged by these results, selenite broth has an advantage over DCA though both have a high degree of selectivity individually. The highest percentage of positive isolations was obtained with the combination of selenite broth and DCA, though this was perhaps less than expected. However, a rough assessment of the amount of growth of the salmonellae on the solid media (Table 2) showed that the most abundant growths were obtained from selenite broth, particularly in combination with DCA.

Table 2. *A comparison of the distribution of amounts of growth of salmonellae obtained from* 139 *positive samples of faeces by direct plating on DCA*, and those obtained by streaking on MA and DCA after preliminary enrichment in SB or MPB*

Isolation procedure	Numbers of cases with growth arbitrarily rated as				
	None	Scanty	Light	Moderate	Heavy
DCA* direct	45	18	15	18	43
SB, MA	13	8	8	36	74
SB, DCA	7	7	9	26	90
MPB, MA	109	4	6	10	10
MPB, DCA	21	11	17	33	57

* DCA, deoxycholate-citrate agar; SB, selenite broth; MA, MacConkey's agar; MPB, mannitol-peptone broth.

The growth of bacteria in selenite broth containing different carbohydrates

In this study the growth curves of *Salm. typhi-murium*, *Pr. vulgaris*, *Cit. freundii* I and *E. coli* I were compared in selenite broth with no carbohydrate or with 0·5% (w/v) of lactose, mannitol, or sucrose. Of these carbohydrates *Salm. typhi-murium*

fermented only mannitol and *Pr. vulgaris* only sucrose, while *Cit. freundii* I and *E. coli* I attacked both lactose and mannitol. As shown in Fig. 1, the presence of unfermentable carbohydrate in no case altered the shape of the growth curve. However, fermentable carbohydrates improved growth in all cases except that of *E. coli* I, where there was no effect for the first 8 hr, after which there was a striking fall in the viable count.

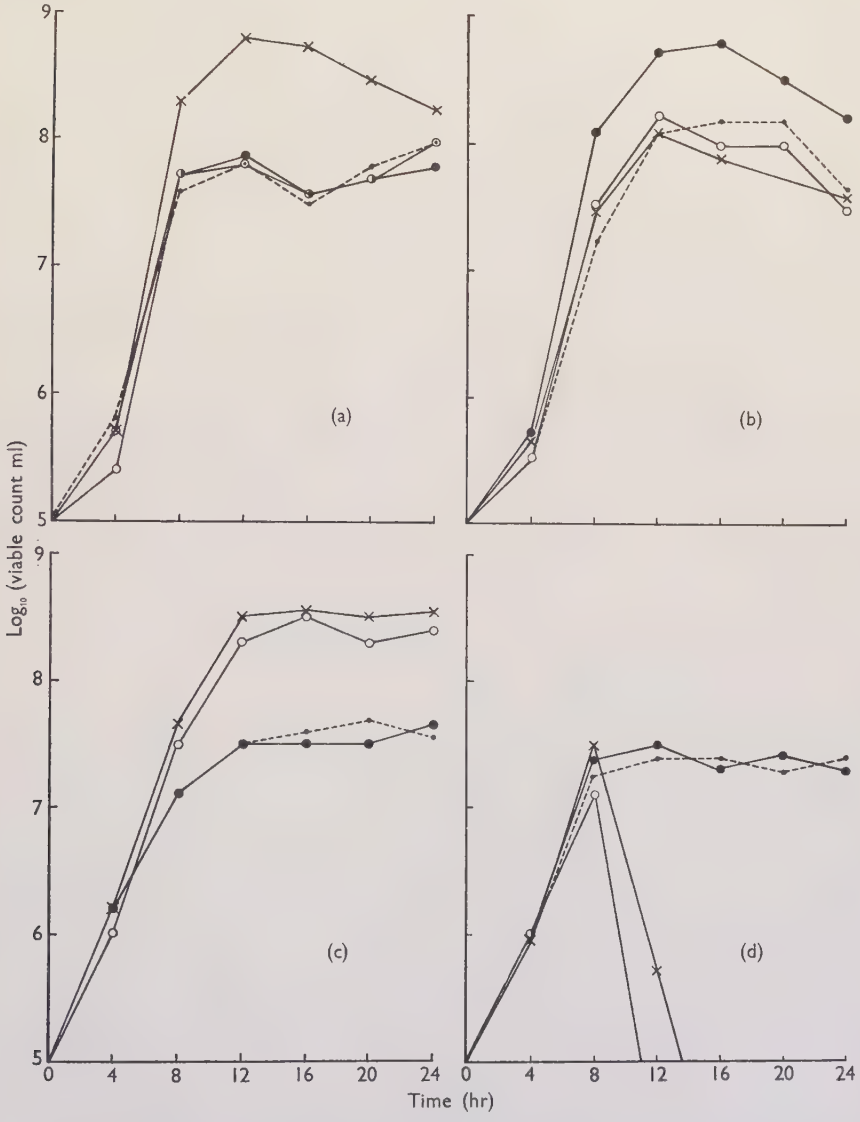

Fig. 1. The influence of carbohydrate on the growth of certain bacteria in Proteose-Peptone selenite broth.
Broken lines, no carbohydrate; open circles, lactose; full circles, sucrose; crosses, mannitol.
(a) *Salm. typhi-murium*, (b) *Pr. vulgaris*, (c) *Cit. freundii* I, (d) *E. coli* I.

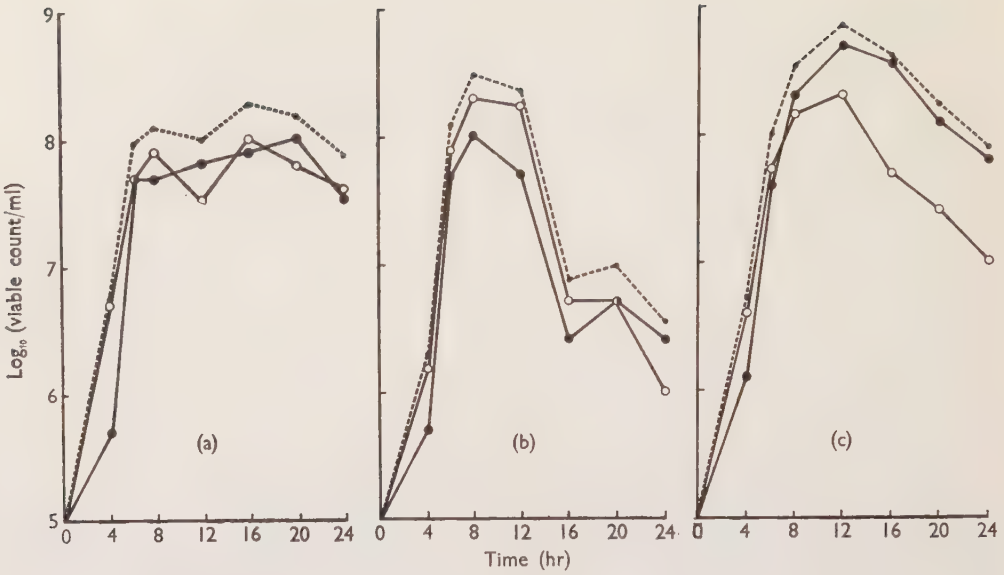

Fig. 2. The effect of mannitol and sucrose on the growth of *Salm. typhimurium* (open circles) and *Pr. vulgaris* (full circles) in mixed cultures in Proteose-Peptone selenite broth. Broken lines, total viable count. (a) No carbohydrate added; (b) mannitol; (c) sucrose.

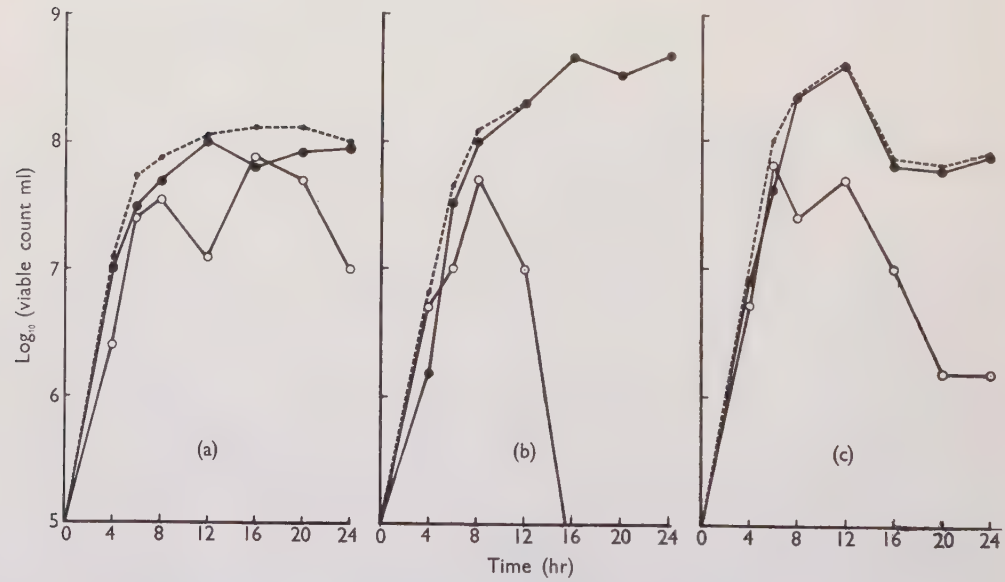

Fig. 3. The effect of lactose and mannitol on the growth of *Salm. typhimurium* (open circles) and *Cit. freundii* I (full circles) in mixed culture in Proteose-Peptone selenite broth. Broken lines, total viable count. (a) No carbohydrate added; (b) lactose; (c) mannitol.

To obtain further information, similar studies were also made using mixed cultures of organisms with different carbohydrates and no carbohydrate. Two combinations of organisms were used, *Salm. typhi-murium* with either *Pr. vulgaris* or *Cit. freundii* I. The inocula were approximately equal, *c.* 10^5/ml of each organism. To enable separate counts of each organism to be made, the viable counts were done on sucrose-peptone agar for the former combination and on lactose-peptone agar for the latter.

With the combination of *Salm. typhi-murium* and *Pr. vulgaris* the two organisms grew equally well with no carbohydrate present (Fig. 2a), but mannitol enhanced the growth of the former while sucrose favoured the latter (Fig. 2, b and c). However, with either mannitol or sucrose present the counts of both organisms fell sharply after 12 hr, this being especially noticeable with mannitol. The combination of *Salm. typhi-murium* and *Cit. freundii* I showed more striking differences. Although the growth curves with no carbohydrate present corresponded fairly closely (Fig. 3a), in lactose selenite broth the former was eventually swamped by the latter (Fig. 3b), while in mannitol selenite broth (Fig. 3c), though *Cit. freundii* I grew more luxuriantly, viable counts of *Salm. typhi-murium* were obtained throughout the whole 24 hr period.

The enhancement of selectivity of selenite broth by gentian violet

Preliminary experiments of the effect of different concentrations of gentian violet in nutrient broth on the growth of different Gram-negative bacilli showed that *Salm. typhi-murium* and *Salm. dublin* grew well in a concentration as high as $5:10^5$, whereas with *Pr. vulgaris* the numbers were greatly reduced with $1:10^5$ and growth was completely suppressed with $2:10^5$. The effect of gentian violet was even more striking with *Shigella sonnei*, growth being almost completely suppressed at $1:10^5$. *E. coli* I was also sensitive, but less so than *Pr. vulgaris*. *Cit. freundii* I and *Klebsiella aerogenes* I had about the same resistance as the salmonellae.

The effect of gentian violet at $1:10^5$ on the growth of a number of organisms in selenite broth was then determined by comparing the growth curves in the presence and absence of the dye. With *Salm. typhi-murium* and *Cit. freundii* I there was only a slight extension of the lag phase, but *E. coli* I was completely suppressed. *K. aerogenes* I, despite a low level of growth in selenite broth without dye, was unaffected. The growth curves of *Salm. typhi*, *Salm. typhi-murium* and *Pr. vulgaris* are shown in Fig. 4. *Salm. typhi* grew well in selenite broth, but the dye had a striking inhibitory effect, mainly on the lag phase. With *Pr. vulgaris* the rate of growth in the log phase was lower in the gentian violet selenite broth and the maximum number of organisms attained in the stationary phase was considerably less.

The effect of $1:10^5$ gentian violet on the growth of a number of *Salmonella* serotypes in selenite broth was also determined. Most of them developed a heavy growth after 18 hr, accompanied by massive reduction of the selenium compound. *Salm. gallinarum* and *Salm. pullorum* grew rather poorly in the selenite broth but were not inhibited by the dye. On the other hand, three laboratory strains of *Salm. typhi* were inhibited, though growth was luxuriant in the dye-free broth. *Salm. cholerae-suis* was completely inhibited in both media.

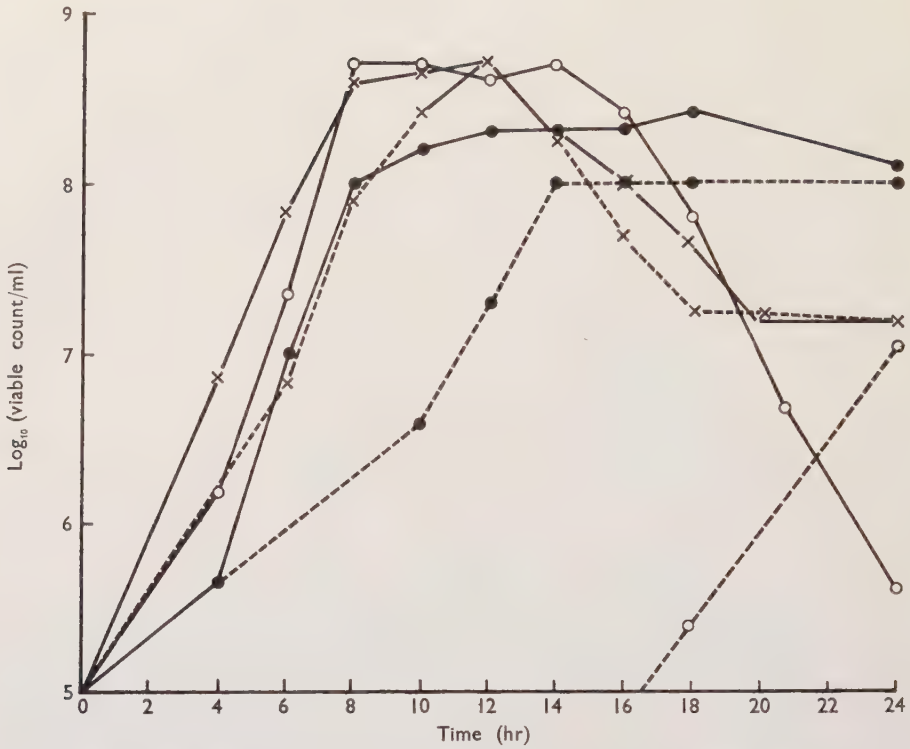

Fig. 4. The effect of $1:10^5$ gentian violet on the growth of certain bacteria in selenite broth.
Open circles, *Salm. typhi*; full circles, *Pr. vulgaris*; crosses, *Salm. typhi-murium*.
Continuous lines, no dye; broken lines, dye present.

An evaluation of selenite broth containing $1:10^5$ gentian violet was undertaken with specimens of faeces sent in for examination for salmonellae or dysenteric organisms. The selenite broth was the medium used routinely in the laboratory at that time and was similar to that used for the isolations recorded in Table 1. Both the dye-free and dye-containing media were inoculated with a loopful of faeces, and after overnight incubation a loopful of the broth was streaked out on MacConkey's agar. As a large outbreak of paratyphoid fever occurred during the period of the investigation, *Salm. paratyphi* B was the predominant serotype found. Of 1,503 specimens examined the total number of salmonella positive stools was 358 but 46 have been excluded because insufficient information was recorded. Of the remaining 312 positive findings 120 were primary isolations, as follows: *Salm. paratyphi* B (74), *Salm. typhi-murium* (32), *Salm. typhi* (6), *Salm. thompson* (7) and *Salm newport* (1). There were more positive isolations with gentian violet selenite broth (300, or 96·1%) than with selenite broth (268, or 85·9%). The standard error of the difference between the percentages, given by the expression $\sqrt{(96{\cdot}1 \times 3{\cdot}9 + 85{\cdot}9 \times 14{\cdot}1)/312}$, was then calculated, and its value found to be 2·24. As the difference between the

percentages (10·2) is more than twice the standard error, the benefit from the use of gentian violet broth, with streaking on MacConkey's agar, is statistically significant. Comparison of the amounts of growth on the MacConkey's agar from the two enrichment media is shown in Table 3. It is evident that in addition to the larger number of positive isolations of salmonellae when the selenite broth contained gentian violet, there was also a strong tendency for the growth to be more abundant.

Table 3. *A comparison of the distribution of amounts of growth of salmonellae obtained on MacConkey's agar after preliminary enrichment in selenite broth with and without* 1 :10^5 *gentian violet*

Dye in selenite broth	Percentages of cases with growth arbitrarily rated as			
	Abundant	Good	Moderate	Poor
Present*	64	21	11	4
Absent	59	17	20	4

* There were 300 positive isolations from cases with dye present and 268 with dye absent.

In view of the fact that gentian violet inhibited pure cultures of *Salm. typhi*, some results obtained from cases of typhoid fever are of particular interest. *Salm. typhi* was isolated from 9 stools with gentian violet selenite broth, but only 7 of these were positive with selenite broth. These paradoxical findings are no doubt related in some way to the modifying effect of faeces.

Discussion

From the growth curves, conclusions that may be generally applicable to the use of selenite broth as a selective medium for the isolation of salmonellae may be drawn. The salmonellae grew more luxuriantly in the presence of a fermentable carbohydrate, but there was a greater tendency for the numbers of organisms to fall sharply after the peak of growth. This decline also appeared to be associated with reduction of the selenite to selenium, which is always greatest in media with a fermentable carbohydrate. With mixed cultures the presence of a carbohydrate which is utilizable by one member may give rise to conditions which affect the viable count of another which may not itself use the carbohydrate. This is strikingly illustrated by the mixed culture of *Salm. typhi-murium* and *Cit. freundii* I in lactose selenite broth (Fig. 3b). *Cit. freundii* I can also grow well on DCA and it, and organisms like it, could cause great difficulty in the isolation of salmonellae. Experimentally, there is no justification for the use of lactose in selenite broth, though the inclusion of mannitol may have some selective value. On the other hand there is evidence (Fig. 3c) that mannitol may not always give an advantage to a salmonella and it is probable that sometimes selenite broth without a fermentable carbohydrate might be the better medium. Possibly comparative tests with and without mannitol in selenite broth in the examination of both foods and faeces would be useful.

It is evident that in selenite broth containing a fermentable carbohydrate better

results might be obtained if the subculture on to solid medium was made before the peak of the logarithmic phase was reached. However, bacteria in foods, especially dried and frozen foods, would most likely be in the lag phase when put into enrichment broth whereas the inocula for the growth curves were of actively growing organisms. The rate at which the food organisms would move out of the lag phase would, therefore, influence the optimal subculture time. The variations of different selenite broths due to different makes of peptone or to different batches of the same make (North & Bartram, 1953) would also influence the optimal subculture time. On the other hand, the type of material introduced into the selenite broth may change the character of an inhibiting selenite broth and promote a rich growth of salmonellae. With faeces and sewage this modifying effect is due, probably, to the presence of large numbers of non-competing organisms rather than to the material itself. Possibly some materials may even increase the toxicity of the medium. The success of small inocula when salmonellae are numerically superior is no doubt due to the diluting out of competing organisms that can grow equally well in fluid or on solid selective media. If salmonellae are present in smaller numbers the chance of isolating them would be very remote. As the type of inoculum might influence the optimal subculture time, and the type of inoculum and its flora the ease or difficulty of isolation of any salmonella from selenite broth, the results with pure, or pure mixed, cultures may be vastly different from those obtained with foods and faeces as inocula.

Selenite broth is commonly used for the isolation of *Shigella sonnei*, which in pure culture is inhibited by gentian violet. This limits the value of the dye in selenite broth for routine examination of faeces. However, with materials that are likely to be heavily contaminated with other organisms, such as sewage effluent and sewer swabs, gentian violet selenite broth might be used with advantage.

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THE EFFECT OF FREEZING AND STORAGE ON THE BACTERIAL FLORA OF PASTEURIZED MILK

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SUMMARY: Experiments were designed to determine the effect of freezing and storage on the bacterial population of pasteurized milk. Samples taken on separate occasions sometimes reacted differently to freezing treatment. Significant changes in bacterial numbers occurred after the frozen milk had been stored for 4–5 months at −12°, and this coincided with definite physical changes in the milk. Known numbers of coli-aerogenes organisms were added to the pasteurized product in order to study their behaviour when frozen and stored at low temperatures. The counts were significantly lower after storage for 4 months.

Mattick (1951) discussed the effect of cold on micro-organisms and quoted the classical work of Haines (1938) which showed −2° to be the most lethal temperature for bacteria in the frozen state; at this temperature 50% of the coagulable protein was precipitated, but at −20° no such change took place. The effect of low temperatures on micro-organisms in relation to dairying was discussed by Davis (1951), who stated that whole milk frozen and stored at −23° could be kept for about 4 months.

The literature on the manufacture and storage of frozen milk has been recently reviewed by Samuelsson, Thomé, Borgstrom & Hjalmdahl (1957), who concluded that in order to maintain the physical stability of the milk it was necessary to carry out rapid freezing and storage at as low a temperature as possible. Fabian & Trout (1943) found no bacteriological reason why frozen pasteurized cream could not be stored for a year at temperatures ranging from −20·6° to −23·3°. Partial or complete freezing of milk or cream samples was unlikely, according to Johns & Berzins (1955), to cause an appreciable change in the bacterial content. Babcock, Roerig, Stabile, Dunlop & Randall (1947) showed freezing and storage of homogenized pasteurized milk in the frozen state to have a tendency to lower the number of bacteria. This decrease was not materially affected by freezing and storage temperatures, by changes in the storage temperature, or by exposure to room temperature for 4 hr. The experiments reported here were designed to determine the effect of freezing and storage on the flora of pasteurized milk which was not homogenized.

Experimental Methods

Samples of milk for treatment were taken at varying periods throughout the year so that a wide variation in the ambient temperature during the time of production on the farm would be included. In this way it was hoped that changes in the bacterial

flora of the pasteurized milk would be encountered. Phosphatase tests were carried out on the freshly pasteurized milk samples to confirm that they had been effectively heat treated. The frozen milk samples were stored at -12° and examined at intervals up to 6 months.

Treatment of milk. Milk pasteurized by the HTST method was bulked and dispensed in 1 pint quantities into polythene bags which were placed in metal moulds and immersed in brine at –28°; the freezing time was 60–65 min. The frozen milk was packed in cardboard containers.

Melting the frozen milk. The polythene covering was first removed and the solid block placed in a sterile glass container immersed in a water bath at 40–42°. Lower temperatures were occasionally used. This normally ensured the melting of the product within 40 min, but when the frozen milk had been stored for over 4 months it required a longer melting time, sometimes up to 70 min.

Colony counts. The colony count of each sample of liquid milk was determined at 3°, 22°, 30° and 37° by plating various dilutions on Yeastrel milk agar (YMA) and then incubating for periods of 21 days at 3°, 5 days at 22°, 3 days at 30° and 2 days at 37°. Duplicate plates were prepared for each dilution. Portions of 1 ml of undiluted milk and of the 10^{-1} dilution were also plated for coli-aerogenes bacteria on violet red bile (VRB) agar, incubated for 18–24 hr at 30° and the numbers of dark red colonies over 0·5 mm in diameter recorded. Similar colony counts on YMA and counts on VRB were done on the frozen product after melting.

Examination of flora present. Comparisons were made of the flora present on the YMA plates at 30° from the milk before and after freezing. After incubation for 3 days every colony on a plate from a suitable dilution was taken and purified at 30°. The morphology of the cultures and their reaction in litmus milk incubated for up to 10 days at 30° were studied. When the bacteriological examinations were completed the samples were held at room temperature for 24 hr so that any visible changes in the milk could be noted.

Viability of coli-aerogenes organisms when frozen in milk. To determine this, four types, *Escherichia coli*, *Klebsiella aerogenes*, and *Citrobacter freundii* types I and II, were used. The growth on an 18 hr Yeastrel milk agar slope was dispersed in Ringer's solution and the approximate number of bacteria present was determined using Brown's opacity tubes. A controlled number was inoculated into bulked pasteurized milk which did not show coli-aerogenes bacteria in 1 ml, and the actual number present in the milk was determined by plating on VRB agar. The number inoculated varied from 10^1 to 10^4/ml. The milk was then frozen, as previously described, and the count on VRB determined immediately after freezing and at varying intervals after storage at –12°.

Presence of Pseudomonas flourescens During the investigation it was noted that some colonies present on YMA plates incubated at 3–5° fluoresced under ultraviolet light. It was therefore decided to examine for *Pseudomonas fluorescens* in 1 ml quantities of the unfrozen and frozen product by plating on medium B described by King, Ward & Raney (1954) and incubating at 30° for 4 days. Acidity determinations were carried out on the pasteurized milk and on the frozen samples which had been stored for 4 months.

Significance of results. Analyses of variance of the logarithms of the plate counts were made to determine the significance of treatment effects. Since the agreement between duplicate plates was very high, the mean values were always used in the analyses. The significance of treatment and sample difference was tested using the treatment × sample interaction as the lesser mean square. The significance of this interaction was tested using the variation within similarly treated samples as the lesser mean square. The Tables show the treatment means for some of the colony counts, the figures having been reconverted from the logarithmic means.

Results

Heat treatment of milk. The phosphatase test results, using the Aschaffenburg (1953) technique, showed that all the samples had been effectively heat treated.

Absence of contaminants in milk samples. All the freshly pasteurized and stored frozen samples were found to have no coli-aerogenes bacteria present in 1 ml on VRB at 30°. No fluorescent colonies were present when 1 ml quantities were incubated at 30° on the special medium of King, Ward & Raney (1954). Nevertheless some fluorescent colonies were detected on a few samples which were plated on YMA and incubated at 3–5° for 21 days.

The acidity of all samples was within the normal range of freshly pasteurized milk and this was also so with those samples which at 4 months showed definite signs of physical change.

Colony counts at 3–5°. These were consistently low in all samples, and rarely exceeded 100/ml. With the majority of unfrozen pasteurized samples the mean count was below 10/ml. There was a tendency for an increase in the colony count in the stored frozen samples. Where the numbers were low and the colonies small the counts were considered to be unreliable because of the physical changes which had occurred in the milk. Accordingly, the colony counts at 3–5° were not subjected to statistical analysis.

Effects on the YMA colony counts at 22°, 30° *and* 37°

The effect of freezing. An analysis of the colony counts at 22°, 30° and 37° of the pasteurized milk and the same milk within 3 hr of freezing was made on samples taken on 13 different occasions. The appropriate figures are shown in Table 1.

Table 1. *The effect of freezing at* −28° *on the mean colony count of freshly pasteurized milk*

Incubation temperature (°C)	Mean colony count* (10^3/ml)		Grand mean
	Before freezing	After freezing	
22	76	83	80
30	61	64	62
37	24	18	21

* Means of 13 determinations.

The difference between the means for the pasteurized and frozen samples was not significant at 30°; it was significant ($P < 0{\cdot}05$) at 22° and at 37°, there being a rise at 22° and a fall at 37°. At 22° and 30° the variation between samples taken on the 13 different occasions was highly significant ($P < 0{\cdot}001$), and also highly significant ($P < 0{\cdot}01$) at 37°. The counts at 37° showed a highly significant interaction between treatment and date of sampling ($P < 0{\cdot}001$), indicating a tendency for the samples taken on different days to have reacted differently to freezing. The interaction was not significant for counts at the other two temperatures.

The effect of storage. The effect of storage at −12° for 5 days on the total count at 22°, 30° and 37° was investigated on samples taken on four different dates (Table 2). The mean count at 37° was significantly lower ($P < 0{\cdot}001$) than the counts at the other two temperatures. Over the 5 day period, storage had no effect on the YMA colony count at any of the temperatures. The differences between the counts on consecutive days were not significant; but differences between the samples taken on different dates were highly significant ($P < 0{\cdot}01$). The interaction between storage treatment and date of sampling was not significant.

Table 2. *The effect on the mean colony count on YMA of frozen pasteurized milk of storage for 5 days at* −12°

Incubation temperature (°C)	Mean colony count* (10^3/ml) after storage for (days)					Grand mean
	1	2	3	4	5	
22	77	72	71	77	83	76
30	68	69	71	76	74	72
37	30	34	31	31	32	32

* Means of determinations on four separate occasions.

The effect of storage at −12° for 6 months on the YMA colony count at 22°, 30° and 37° was studied, using samples taken at monthly intervals on two different dates (Table 3). The results showed that a significant drop ($P < 0{\cdot}01$) occurred at all temperatures at the end of the 4th month. The method of melting the samples did not affect this, similar results being obtained when lower melting temperatures were used. A very obvious change in the physical condition of the

Table 3. *The effect on the mean colony count on YMA of frozen pasteurized milk of storage for long periods at* −12°

Incubation temperature (°C)	Mean colony count* (10^3/ml) after storage for (months)							Grand mean
	0†	1	2	3	4	5	6	
22	91	90	79	71	57	51	45	69
30	74	79	70	70	50	48	40	62
37	33	37	50	31	16	9	10	27

* Based on samples taken on two separate occasions.
† After storage for 1 day.

sample coincided with the drop in bacterial numbers, but this did not affect the accuracy of the counts, as the usual high agreement between duplicates was obtained.

The effect of freezing and low temperature storage on the viability of coli-aerogenes bacteria

The effect of freezing. On nine occasions a bulk of freshly pasteurized milk, from which coli-aerogenes organisms were subsequently shown to have been absent, was inoculated with representative organisms of this group. On three occasions *E. coli* I was used, the inoculum varying from 435–7,850/ml. On two occasions *K. aerogenes* I was used, with inocula of 250 and 7,800/ml. On two occasions *Cit. freundii* I was used, at the rate of 270 and 410/ml. For the remaining two investigations *Cit. freundii* II was used, at 20 and 365/ml. Immediately after freezing the count on VRB agar at 30° was determined. There was no significant difference between the counts for the unfrozen and frozen milk samples, but there was a highly significant difference (P <0·001) between samples taken on the nine occasions. The interaction between treatment and sampling date was not significant.

The effect of storage. Samples from the same inoculated bulk of milk were examined after storage at –12° for 5–6 weeks and after storage for 17–19 weeks. The difference between samples taken on different dates was highly significant (P <0·001). The counts at the last period (17–19 weeks) were significantly less (P <0·001) than the counts for the first period (fresh frozen) and those for the second period (5–6 weeks), which were only slightly less than the initial counts. The interaction between sample and treatment was highly significant (P <0·001), showing that samples taken on separate occasions reacted differently to the storage periods.

The effect of freezing on the flora of pasteurized milk

Eight samples were examined. They yielded a total of 297 colonies of which 144 were from the unfrozen pasteurized milk and 153 from the unstored frozen product. The reactions of these organisms in litmus milk, taken up to 10 days incubation at 30°, are given in Table 4, which shows that the distribution of the different litmus

Table 4. *The effect of freezing on the flora of pasteurized milk, as shown by the litmus milk reactions of organisms isolated on YMA at* 30°

Reaction in litmus milk at 30°	Percentages of organisms*	
	Before freezing	After freezing
Slight acid	21·5	24·8
Acid	20·1	19·6
Acid and clot	9·0	13·7
Acid, clot and bleaching	38·9	27·5
Alkaline	3·5	6·5
Proteolytic	3·5	4·6
None	3·5	3·3

* Based on 144 cultures from the unfrozen and 153 from the frozen milk.

milk reactions of the organisms from both types of milk was closely similar. The two floras were also morphologically similar. The flora of the freshly pasteurized milk was normal. It contained only about 1% of sporeforming rods.

Discussion

The milk when melted was normal in every respect after storage at −12° for 6 weeks. After this period all the samples examined showed a very slight physical deterioration, the melted milk having a slightly flocculent appearance. This disappeared readily when the milk was heated and shaken. The change in the physical condition was progressive with increased time of storage and it became increasingly difficult to obtain a product of normal appearance. At 17 weeks the milk could still be reconstituted by boiling and shaking, but at 18 weeks this was impossible even when these treatments were prolonged. The acidity of such milk was unchanged, but no attempt was made to study the nature of the change.

The normal flora of pasteurized milk was unaltered by freezing and limited storage, and when contaminants of the coli-aerogenes group were added their numbers were not decreased by freezing. The changes in bacterial numbers which occurred between the 3rd and 4th month (Table 3) coincided with the appearance of a definite physical change.

In nearly all instances, differences between samples taken on different days were highly significant and often more important than differences between treatments. Moreover, in some instances, there was a significant interaction between treatment and sample, indicating that samples taken on different days reacted differently to freezing and storage. These aspects of the subject are being investigated further.

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THE OCCURRENCE OF *BACILLUS CEREUS* IN MILK AND ON DAIRY EQUIPMENT

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SUMMARY: In a survey of milk obtained from individual farms and creameries, 87% of samples were found to contain fewer than one cell of *Bacillus cereus*/ml. The remaining 13%, although they all yielded low counts, showed that the organism tended to occur in milk received from particular farms and creameries. On investigating sources of contaminated milk, it was found that the organism was derived chiefly from cans which, after being emptied of milk, had been allowed to stand for a long time before being washed properly and 'sterilized'. The vegetative cells of *B. cereus* were shown to form spores readily in thin films of diluted milk, such as may occur in a rinsed can.

The importance of *Bacillus cereus* in the deterioration of pasteurized milk has been receiving increasing attention, more especially since Stone & Rowlands (1952) showed that it produced the condition known as 'broken' or 'bitty' cream. This fault has proved exceedingly difficult to control because a comparatively light seeding with *B. cereus* is apparently dangerous and, as Billing & Cuthbert (1958) showed in an extensive investigation of the problem, counts of the spores of this organism in milk bear little relation to the degree of contamination with other types of bacteria.

The object of the work described below was to secure further information concerning the occurrence of *B. cereus* in milk and on dairy equipment. The enquiry was conducted in an area where 'bitty' cream has not attracted attention. Importance was attached to the use of a quantitative method by which vegetative cells as well as spores could be estimated. A plating medium which appears to fulfil these requirements has already been described (Donovan, 1958).

Methods

Milk samples. The samples examined were mainly taken at two large Scottish creameries, A and B. These creameries pasteurized milk, which they received in cans from individual farms and in road tankers from collecting depots. At each creamery, samples were taken from different parts of the plant, from the road tankers and from the weigh tank (the latter representing the bulked milk of individual farms). Sampling was also done on farms and at collecting depots. All samples were taken by sterile dipper or through cocks or loosened joints into sterile bottles of *c.* 120 ml capacity.

Treatment of samples. The samples were held in a refrigerator until they were examined. From each sample two 10 ml subsamples were taken into $6 \times \frac{5}{8}$ in. tubes. One tube was pasteurized at 63° (±0·5°) for 30 min; the other was not so treated.

Examination of subsamples. The raw subsamples were pour-plated with egg yolk-inhibitor agar and the pasteurized subsamples with plain egg yolk agar. Both media

have been described previously (Donovan, 1958). The plates were incubated at 30° for 18 hr.

Examination of rinses and swabs. Poured plates of yolk-inhibitor agar were incubated for 18 hr at 30°. Swabs were placed into 25 ml of sterile water at the time of sampling, and were examined by plating the water.

Tests of conditions which influence spore formation. Vegetative cells were obtained from cultures prepared at 22° in glucose-peptone water (glucose, 0·5% w/v; peptone, 0·5% w/v), repeated transfers of young cells through this medium having been made for at least four days previously. The absence of spores was confirmed by plate counts after heating for 30 min at 65°. The cells were washed four times in glass-distilled water and used at the required dilution for inoculating the test medium. After incubation the culture was diluted and divided into two parts, one of which was held at 65° for 30 min and pour-plated for a spore count. The unheated portion was plated for a total viable count. In both cases the medium used was a nutrient agar (peptone and beef extract, each 0·5% w/v), with 0·1% of soluble starch, and incubation was at 30° for 48 hr.

Results

Milk samples from different sources

Table 1 gives a summary of the results of the examination of 411 samples taken at creameries A and B. It can be seen that 13% contained one or more *B. cereus*/ml. Of these samples, 82% were detected by plating raw milk and 44% by plating the laboratory-pasteurized subsamples. However, the counts were so low that the difference is likely to be within the error of the method, and does not prove that vegetative cells occurred more frequently than spores. The average count in the positive samples was only 4/ml.

Table 1. *The occurrence of* B. cereus *in milk from farms, collecting depots and creamery plant*

Source of milk	Sampling creamery	Total samples	Samples yielding *B. cereus* in 1 ml	
			No.	%
Farms	A	61	13	21
	B	219	14	6
Collecting depots	A	43	10	23
	B	4	0	0
Creamery plant	A	60	13	22
	B	24	2	8
All sources	A	164	36	22
	B	247	16	7
	A+B	411	52	13

Despite the small counts certain trends were apparent. It was noticeable that for each of the three sources of milk—single farms, depots and creamery plant—the samples taken at creamery A contained the bacillus more frequently than did those

taken at B. This suggested some influencing factor associated with creamery A. Consideration of the results for all the single farm supplies, irrespective of the creamery at which the sampling was done, showed that positive results were obtained with milk from 11 of the 111 farms. Similarly, the results for all the collecting depots were examined. Milk was brought by road tanker from 11 depots, of which 3 were responsible for 71% of the positive results.

This preliminary survey suggested a closer investigation of the methods in use at the farms and collecting depots with which *B. cereus* appeared to be associated, and also at creamery A.

Detailed investigations at farms

Visits were made to five farms, of which three (farms B, L and M) had consistently shown small counts of *B. cereus* in milk delivered to a country creamery T. The other two farms (S and W) had consistently given negative results. In each case the equipment was examined in detail by means of rinses, swabs and milk samples, and the results are given in Table 2. Noteworthy counts of *B. cereus*, where these occurred, were associated with cans and the articles used in cleaning and sterilizing them.

Table 2. *The occurrence of* B. cereus *on the equipment of five dairy farms*

Source of sample	Nature of sample	*B. cereus* count(/ml)* in individual samples from farm				
		B	L	M	S	W
Clusters	500 ml rinse	<1(4)	<1(4)	3, <1	—	<1(4)
Unit buckets	500 ml rinse	<1(3)	<1(4)	<1(3)	—	<1(4)
Milk lift	500 ml rinse	<1	—	—	—	—
Cooler	Swab	<1	<1	<1	—	<1
Cans (steamed)	500 ml rinse	142, 118, <1(4)	>200, <1	46, 3, 1	<1(2)	<1(4)
Can brushes	Rinse	9, <1	51	1, <1	150, 5	<1(2)
Cloth, can-washing	Rinse	>38	>200	17	<1	—
Water before use for can washing	—	<1	94, 2	1	—	—
Steaming stool	Swab	>200	—	—	—	—
Unit buckets	First milk into	<1(3)	<1(4)	<1(2)	—	<1
Cooler	First milk over	<1	—	<1	1	<1
Cans	Milk	3, <1(2)	30, 13, 1, <1	4, 1, <1	<1(2)	<1(2)

* Figures in brackets show the number of samples yielding <1/ml when more than one sample gave that result.

Investigation of the histories of the cans revealed that after being tipped at the creamery they were rinsed with cold water and left upright on the platform until the following day, when they were collected by their owners. When milky water taken from cans after they had stood overnight at the creamery was examined, four out of eight showed high counts of *B. cereus*. The cans which gave the data shown in Table 2 had been steamed on the farms. This may be taken to indicate—even though the treatments were inadequate—that the spore stage had been reached.

Detailed investigation of equipment and methods at creamery A

Table 3 contains the result of examinations of the plant. The organism appeared to be randomly distributed through the plant, there being no suggestion of an increase at any particular point. These results thus gave no hint of the reason why *B. cereus* had been found more frequently at creamery A than at creamery B. However, reconsideration of the earlier findings revealed the fact that a higher frequency had been shown also by the individual farm milks supplied to creamery A. The farm supplies had been sampled before the milk made contact with the plant, and it seemed that the only operation at the creamery that could affect all the producers was the can washing, in which all cans, immediately after being tipped, were put through a mechanical washer and sterilizer. On several occasions rinses from cans selected at random after they emerged 'sterile' from the washer were examined for *B. cereus*, and of 22 cans thus tested 14 proved to be contaminated, the average count of the organisms being of the order of 16×10^3/can. The 'sterilization' process in the washer was obviously incapable of destroying the spores of *B. cereus*.

Table 3. *The occurrence of* B. cereus *in different sections of the plant of a creamery*

Source of samples	Occasions of sampling	Samples positive in 1 ml		
		No.	%	Mean count (/ml)
Weigh tank	5	0	0	—
Before 1st pump	6	1	17	1
After 1st pump	7	1	14	6
Storage tanks	13	6	46	2
Before 2nd pump	4	1	25	1
After 2nd pump	8	1	13	3
Balance tank	9	3	33	2
Cooler exit	8	0	0	—

Further inquiry into the methods in use at creamery A revealed that in addition to the farmers' cans, certain cans belonging to the creamery were put through the washer. These cans were used for sending pasteurized milk to schools, hotels and restaurants, and as owner, the creamery was responsible for cleaning them. Thus a rinse with cold water was usually the most that any can received before being returned to the creamery. Moreover, the arrangements were such that periods up to a week elapsed before the cans were collected. Examination of watery residues taken from 80 of the cans upon their return to the creamery showed that 48 contained *B. cereus*, the average count being 26×10^3/ml of residue. Although these cans seemed a likely source of contamination of the washer, it was impossible to obtain direct evidence that this was so. Another way in which the creamery cans could have had an effect was in their use for the overnight storage of returned milk. This milk was tipped into the cans from returned bottles in the evening and repasteurized first the following morning. Large volumes were involved at certain times of the year.

Conditions which influence spore formation

It seems clear from the foregoing results that cans are an important source of contamination of milk by *B. cereus*. The fact that this was true of cans which had undergone a form of 'sterilization' suggests that the organism was in the spore stage, for the vegetative cell has been shown in this laboratory to be destroyed within a few minutes at 63°. Moreover, the common observation that this bacillus produces faults chiefly in pasteurized milk is a further indication of the importance of the spore. It was of interest, however, to examine some of the conditions which might affect the process of spore production.

There is general agreement that most species of *Bacillus* do not readily form spores in milk (see for example Grinsted & Clegg, 1955). That this may be due in part to inadequate supplies of oxygen seems possible, for much evidence, applying to several species, shows that abundant aeration is essential to a maximum production of spores (Leifson, 1931; Brunstetter & Magoon, 1932; Knaysi, 1945; Roth & Lively, 1956; and others). In the present work, when the aim was to provide sufficient oxygen for spore formation, stationary thin films of medium were used. The aeration obtained in this way appeared to be comparable to that in shaken flasks. The medium, after inoculation with vegetative cells (see Methods), was pipetted in 1 ml amounts into a series of $6 \times \frac{5}{8}$ in. tubes with rubber stoppers. The tubes were incubated in the horizontal position, and at intervals the entire contents of each of two tubes were examined. Each 1 ml culture was diluted 1:10 and thoroughly shaken as the first step in making spore and total viable counts.

A reduction of the nutrient concentration in media has been found to promote spore formation in certain species of *Bacillus* by Williams (1930), Brunstetter & Magoon (1932), Bayne-Jones & Petrilli (1933) and Knaysi (1945). The effect on *B. cereus* of diluting milk was therefore examined, trials being made with 13 strains which had been isolated from milk. Sterile skim milk and the same medium diluted 1:50 with glass-distilled water were inoculated with vegetative cells and incubated as thin films at 30°. There was a strain to strain variation in the time at which spores appeared and in the final ratio of spore count to total viable count. There was also a variation in the performance of a single strain in different tests, indicating that the conditions were not completely uniform. Nevertheless, dilution of milk was convincingly shown to promote spore formation. In the undiluted milk, spores never reached 1% of the total plate count at 48 hr; in the milk diluted 1:50, the proportion of spores varied between the limits of 70% at 48 hr and 100% by 24 hr.

The influence of aeration on spore formation in undiluted milk and in milk diluted 1:50 was next examined. The results obtained at 24 hr when incubating at 30° with two grades of oxygen supply are given in Table 4. It is clear that while milk was an unsuitable medium for spore production, diluted milk in thin films provided excellent conditions for the process. This appears to be a sufficient explanation for the appearance of spores in milk cans. Given a suitable temperature and sufficient time for the completion of the vegetative phase, many spores could be formed in traces of milky water.

Several workers (Foster & Heiligman, 1949; Charney, Fisher & Hegarty, 1951; Curran & Evans, 1954; Weinberg, 1955) have found that spore formation in some

species of *Bacillus* may be promoted by the addition to nutrient media of trace amounts of certain metals, notably manganese, and it seemed possible that metal ions derived from cans might have some importance in the case of *B. cereus*. However, in tests in which manganese and potassium were added to milk and diluted milk, no effect of these elements was detected.

Table 4. *The influence of aeration on spore formation by* B. cereus *incubated for 24 hr at 30° in diluted and undiluted milk*

Degree of aeration*	Milk	Number of organisms/ml		Proportion of spores (%)
		Total viable	Spores	
Good	Diluted	35,000,000	37,000,000	100
	Undiluted	820,000,000	180,000	0·02
Restricted	Diluted	11,000,000	79,000	0·72
	Undiluted	120,000,000	1,200	0·001

* Good, 1 ml of medium in a horizontal $6 \times \frac{5}{8}$ in. tube. Restricted, 5 ml in a similar but vertical tube.

Discussion

The results presented above of the examination of milk for the occurrence of *B. cereus* were derived from a relatively small survey; indeed, the positive results were obtained from only a small proportion of the samples examined. Nevertheless, certain trends were detected. On following these indications, milk cans were found to be the chief source of the organism. When spore formation by the bacillus was investigated, it was shown to occur profusely under conditions similar to those in a rinsed milk can.

From the evidence collected, one way at least can be suggested in which contamination of freshly produced milk with *B. cereus* may occur at the farm. This involves a cycle, with the treatment of cans as the critical factor. Spores are formed readily in milk much diluted with water under conditions of good oxygen availability, and these conditions may be provided when cans have been rinsed with water after milk has been tipped. If the organism is present, the outcome is likely to be decided by the temperature and by the time that elapses before the can receives further attention. The final treatment applied to the can will depend on the farmer and on the facilities available to him, but a development of spores would increase the difficulty of achieving adequate sterilization. At some farms a consistent failure to destroy spores is to be expected, and in such cases a gradual increase in the contamination of the can is likely to occur.

A large creamery may apparently disseminate the organism after it has multiplied elsewhere. In the case investigated a particular combination of circumstances seemed to be leading to a spread of contamination among cans in the mechanical washer. However, it is significant that, in this instance also, the can was the central factor.

If the findings of this enquiry should apply widely there would appear to be no insuperable obstacle to ensuring that no raw milk is heavily contaminated with *B. cereus*. Judging by the results obtained, and also those of Billing & Cuthbert (1958), the organism has little ability to reach the spore stage on most dairy equipment at the farm. The important source of contamination of raw milk appears to

be the milk can which, after having been emptied and rinsed, is kept in a warm atmosphere until spores have formed. The same general principles may well apply to milk bottles, from which pasteurized milk could be contaminated.

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THE INVESTMENT VALUE OF CONTINUOUS CULTURE

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SUMMARY: Comparisons are given of the output of sorbose from 3 litre cultures of *Acetobacter suboxydans* grown in a sorbitol medium in batch and continuous culture. An estimate is given of the investment value of a continuous process of sorbose production relative to the batch process.

There has been no published quantitative appreciation of the commercial value of continuous as opposed to batch microbial culture, and in discussions on the subject opinion has not always been distinguishable from fact. The most reasoned comment, and the most favourable, is probably that of Maxon (1955) who wrote: 'Although the limitations of continuous culture are recognized, they are not in every case insurmountable. In the simpler fermentations the method has proved its worth. As our experience increases it is probable that more and more fermentation products will be made in this manner.'

Among the advantages of continuous culture, relative to batch, is the reduction in the size and cost of equipment necessary to produce a given output. Further, running costs for items such as steam for sterilization, air, and electrical power for agitation will be less. Cost of supervision will also be less, because recurring operations such as seed preparation and vessel sterilization are eliminated or reduced in frequency. In addition a continuous process is more adaptable to automatic control, with a prospect of further savings in labour costs. Maxon also argued that since continuous culture makes possible both continuous medium sterilization and continuous processing, savings which result from such consequences can be credited to continuous culture. An advantage on which it is not easy to set a value is that steady state continuous operation should result in a product more uniform, in quality and quantity, than one produced by batch methods.

Several advantages have been claimed for batch processes. The first arises from an axiom in production engineering that batch equipment is more adaptable than continuous equipment to changes in programme and that it is unwise to install any continuous plant unless a long period of production at a steady rate is foreseen. However, flexibility has been introduced into continuous production of many products by using a number of small units and adjusting the number in use to the rate of output required. The same method is possible with biosynthesis and it can be added that a culture vessel designed for continuous work can also be used for batch, though the reverse does not apply. It is also said that batch culture is best because a lower standard of maintenance can be accepted, since repair work can be done at

the close of each batch. Likewise, it is said that secondary infection is likely to be less troublesome in batch culture because the equipment is sterilized at each cycle. These advantages no doubt apply if relatively crude equipment and techniques are tolerated: but we believe the argument is without force since we find that equipment of no better standard than that in common use in low-pressure chemical processes, combined with not-above-average operating standards, is entirely adequate for continuous culture. Theoretically the yield per unit of substrate is higher in batch than in continuous culture, but in a correctly conducted continuous culture (where substrate concentration is limiting growth) this difference will not be significant. We do show later that appreciable quantities of unconverted substrate can be tolerated in continuous culture and the product still be cheaper than that made by a batch culture where substrate is completely utilized. Where the situation involves the problem of genetic stability it is undoubtedly true that, as an immediate solution, it is better to use batch culture because the parent strain can thereby be continually renewed. We would, however, quote an authoritative view (that of Dr. Werner Braun, expressed privately to Dr. D. W. W. Henderson) that if the challenge to effect genetic stability is accepted, one probable solution is to grow in steady state continuous culture.

It is also argued that if the culture is multistage (e.g. penicillin production), then batch culture is best because the various stages can be carried out successively in one and the same vessel. On the other hand a continuous culture needs to be carried out in a series of as many vessels as there are stages. Thus greater complexity will result and the saving in capital cost will also be less than in a single stage culture. However, experimental evidence from these laboratories (Pirt, pers. comm.) is showing that continuous production of penicillin is possible in a multistage continuous process at an increased rate of output relative to that in batch equipment of comparable size. The results lead him to believe that this increase will be reflected in increased profitability if conducted on an industrial scale. These arguments tend to show that continuous culture allied to technological and scientific improvements will ultimately show considerable advantage over batch culture. However, if one is prepared to install plant of no worse standard than that commonly used in many chemical processes, and to gain by practice the 'know-how' of overcoming secondary infection, then if a genetically stable single stage culture process is selected it follows that commercially the continuous process will show an immediate advantage over the batch.

It was to test this premise that we carried out the present work, which deals with the conversion of sorbitol to sorbose by *Acetobacter suboxydans* in batch and continuous culture. This process was selected because, among the established methods for the microbial production of organic chemicals, it appears to have good prospects of success in single stage continuous culture, without requiring any research into the microbiology of the process. The cost comparison must also be of interest because the industrial significance of the process is not likely to suffer by the advent of a chemical synthesis, although there is a proposal for an alternative microbial route to vitamin C, for which sorbose is a starting point (Folkers, 1956). Finally, the large amount of published information about the process (Damodaran & Subramanian,

1951; Lockwood, 1954) makes its selection as the subject for a comparison of batch and continuous culture even more suitable. In the paper, output and yields are compared and estimates made of the profitability of the two processes.

PART I. COMPARISON OF OUTPUT RATES

Theoretical

The relation between growth and product formation in a continuous culture. This has been discussed by Maxon (1955), who recognized two classifications: product formation associated with growth and product formation not associated with growth. The sorbose fermentation approximates to the former, which Maxon defines as the situation where a substrate, in this case sorbitol, limits the rates of both processes simultaneously and to a similar extent. When a given substrate is limiting growth, and when growth and product formation are synchronous, product formation can be analyzed according to the theory developed for bacterial growth. It may then be described in terms of the maximum growth rate constant (μ_m), the saturation constant of the limiting substrate ($\boldsymbol{K}_s$); and the product yield constant ($\boldsymbol{Y}_p$, the weight of product formed/weight of limiting substrate used) which replaces the bacterial yield constant ($\boldsymbol{Y}$, the weight of bacteria formed/weight of limiting substrate used). The symbols are those used previously by Herbert, Elsworth & Telling (1956).

When a given substrate is the growth limiting factor. If the concentration of a given substrate has been correctly selected to make it a growth limiting factor, then in batch culture at sufficiently low concentrations of bacteria where aeration is adequate and where hydrogen ion concentration is not inhibitory, it is an experimental fact that after a lag phase growth is exponential until almost all the substrate has been used up, after which the growth rate declines and reaches zero when the substrate is exhausted. From these facts Monod (1942) has postulated that at time t, the relation between specific growth rate (μ) and substrate concentration (s) is given by

$$(1/x.)\,(\mathrm{d}x/\mathrm{d}t) = \mu = (\mu_m s)/(\boldsymbol{K}_s + s) \qquad (1)$$

where x is the bacterial concentration, μ_m is the growth rate constant (i.e. the maximum value of μ, which is reached when $s \gg \boldsymbol{K}_s$) and $\boldsymbol{K}_s$ is a saturation constant numerically equal to the substrate concentration at which $\mu = \frac{1}{2}\,\mu_m$. The recognition of the exponential phase ($s \gg \boldsymbol{K}_s$), and the declining phase ($s \simeq \boldsymbol{K}_s$ to $s=0$), may be used to test that a substrate is growth rate limiting. Perhaps the best way to test whether, at a certain initial concentration, a given substrate is the growth limiting component is to add an additional quantity and then to compare growth in batch culture at the two concentrations. If the effect of adding more substrate is to extend the period of exponential growth then clearly at the first concentration the given substrate was the growth limiting factor. If not, some other reason must be looked for, and the above test can amount to a check that aeration and control of pH value are adequate; or, when high bacterial concentrations are the aim, that trace elements are plentiful.

When growth and product formation are associated. By definition this means that in any interval of time ($\Delta\, t$) the ratio of product ($\Delta\, p$) to bacteria ($\Delta\, x$) is a constant.

In the general case, whether growth is exponential or not, $dp/dt=k(dx/dt)$, where k is a constant. When growth is exponential $dx/dt=\mu_m x$, and if also, as above, $p/x=k$, then it follows that $dp/dt=\mu_m p$. Thus the slopes of the graphs of log x and log p plotted against time will not differ statistically and will be equal to μ_m.

Equations for product formation. If the culture conforms to the highly simplified assumptions made above it is apparent that the steady state value of p, designated as $\hat{p}$, will be obtained by substituting $\hat{p}$ for $\hat{x}$ and $\boldsymbol{Y}_p$ for $\boldsymbol{Y}$ in equation (10) derived by Herbert *et al.* (1956). Thus

$$\hat{p}=\boldsymbol{Y}_p\left\{s_R-\boldsymbol{K}_s\left(\frac{\boldsymbol{D}}{\mu_m-\boldsymbol{D}}\right)\right\} \tag{2}$$

where $\boldsymbol{D}$ $(=f/v)$ is the dilution rate (f being the medium flow rate and v the volume of culture in the vessel) and s_R is the initial concentration of substrate. Equations for output ($\boldsymbol{D}\hat{p}$), dilution rate for maximum output ($\boldsymbol{D}_M$), and product concentration at maximum output ($\hat{p}_M$) are obtained by similar substitutions in equations (13), (14) and (15) respectively in the same paper.

Calculation of oxygen solution rate. For the purpose of designing the culture vessel aeration system, the required aeration capacity can be estimated by assuming that oxygen is used only to convert sorbitol to sorbose. The subsequent work has shown that this is but an approximation and that of the total oxygen consumption some 16% is used to make CO_2. Assuming the specific reaction rate for sorbose formation to be numerically equal to the growth rate constant (μ_m) of the organism, then an estimate of the rate of oxygen consumption dC/dt, in mM/(l.)(hr), in terms of the growth rate constant (μ_m), sorbose concentration (p), and the oxygen equivalent of sorbose (2·8 mM O_2/g sorbose) is given by

$$\frac{dC}{dt}=2{\cdot}8\mu_m p=\left\{\frac{2{\cdot}8\,p}{t_d}\right\}\times \log_e 2 \tag{3}$$

where t_d is the doubling time (in hr) of the organism. At a sorbose concentration of 100 g/l., the maximum level selected in the subsequent work, we get

$$\frac{dC}{dt_{(p=100)}}=280\mu_m=\frac{194}{t_d} \tag{4}$$

From this, when t_d=2·0 and 2·5 hr, dC/dt=97 and 78 mM/(l.)(hr) respectively.

Experimental

Apparatus and Methods

Continuous culture apparatus. The apparatus was similar to that described by Elsworth, Meakin, Pirt & Capell (1956), except for the culture vessel which was that described by Elsworth, Capell & Telling (1958). In addition, instead of using air pressure, the culture medium was metered and transferred by a peristaltic pump (Model T-6, Sigmamotor Inc., Middleport, N.Y., U.S.A.), the reduction gear of which was modified to give a range of 0–25 pulses/min. Using 4·75 mm bore×1·6 mm wall 'Escorubber' translucent silicone rubber tubing, supplied by Esco (Rubber) Ltd., 2, Stothard Place, Bishopsgate, London, E.C.2, the effective metering range was 0·1–1·2 l./hr, using one tube, and 0·6–2·0 l./hr using two tubes. The life of a tube

was about 4 weeks. The impeller was direct driven (1000 rev/min synchronous speed), and measured: A, 63 mm; B, 22 mm; C, 15 mm; D, 4 mm (Fig. 3, Elsworth *et al.* 1956). At a culture volume of 3 l. and an air rate of 3 l./min the sulphite oxidation rate was 174 mM/(l.)(hr), which may be compared with the estimates of O_2 solution rate of 78–97mM/(l)(hr).

Operation. The general methods described by Elsworth *et al.* (1956) were used, with the exception that the culture vessel was sterilized *in situ* by adding formalin to it (Elsworth *et al.* 1958).

Organism and culture medium. The NCIB strain 6723 of *Acetobacter suboxydans*, which is derived from the American strain NRRL B72, was used for the conversion. A working stock culture which was subcultured every 3–4 days was maintained on a solid medium in Petri dishes. This consisted of sorbitol crystalline powder (≥98% sorbitol, Howards of Ilford Ltd., Ilford, Essex), 50 g; Bacto dehydrated yeast extract (Difco Labs. Inc., Detroit, Michigan, U.S.A.), 5 g; agar, 15 g; distilled water to 1 l.; pH 6·6. To prepare a seed, agar slopes in test tubes were inoculated by mass transfer from a stock culture and incubated for 48 hr at 30°. This growth was then dispersed in Tyrode's solution and transferred to 150 ml of liquid medium in a 4 l. bottle. This medium consisted of 100 g/l. of sorbitol crystalline powder and 5 g/l. of Bacto dehydrated yeast extract, made up in tap water and the pH value adjusted to 5·8–6·0. Sterile air saturated at 30° with water vapour was passed at the rate of 0·4 l./min over the surface of the culture. The air connection was made by rubber tube and the bottle was shaken (95 strokes/min; amplitude, 4 in.; motion, reciprocating) at 30° for 48 hr. This 150 ml shaken culture was then used to seed the 3 l. vessel in which the same culture medium was used. Foaming was controlled by manual addition of silicone, which was prepared by dispersing 25 ml of silicone M.S. antifoam emulsion F (Hopkins & Williams Ltd., Chadwell Heath, Essex) in 100 ml of distilled water. This mixture was autoclaved, and if separation occurred it was re-emulsified by heating to 60° and then shaking while cooling under running water. A 0·25 ml dose of this diluted solution added to 3 l. of culture would usually suppress foaming. Over a period, antifoam, measured as diluted solution, was used at an average rate of 0·5 ml/10 l. of culture medium. It accumulated as a solid at the surface of the culture, on the impeller shaft and on the baffles, and very little appeared in the effluent culture.

Observations and measurements. In following the process it would have been ideal to measure the concentrations in the culture of sorbitol, sorbose and bacteria. However, we knew of no suitable routine chemical method of estimating sorbitol and therefore only measured sorbose and bacteria. The concentration of sorbitol in the medium at the start of a culture was calculated from the amount added. To control the accuracy of this addition the refractive index of each batch was measured and compared with values obtained for known mixtures containing 5 g/l. of yeast extract and varying amounts of sorbitol. The satisfactory material balances which were obtained justified the above course. In addition, each batch of medium was tested for sterility, analyzed for total nitrogen (Kjeldahl method) and its pH value determined. Culture samples were tested for reducing sugars, bacterial dry weight, pH value, freedom from contamination and, initially, for nitrogen content (to ensure an

excess). Samples for reducing sugar (i.e. sorbose) were pasteurized at 60° for 1 hr and stored in the refrigerator to await analysis. They were centrifuged to remove bacteria and the supernatant analyzed by the Lane-Eynon general volumetric method (Analytical Method, 1945) using a pure sample of sorbose as standard. Samples for bacterial dry weight were treated with 40% formalin (1 ml of formalin soln/100 ml of sample) and examined in a single cell absorptiometer (neutral grey filter) which was calibrated at each dilution rate against gravimetric dry weight determinations (Wade, 1952). CO_2 in the exit air from the culture vessel was measured by infrared absorption analysis (Telling, Elsworth & East, 1958). The oxygen concentration in the exit air was measured with a Pauling oxygen analyzer (Model S, Arnold O. Beckman, Pasadena, California, U.S.A.) which we modified by adding an inlet port for the admission of the air stream.

Results

Batch culture. This was carried out at atmospheric pressure, at 30°, and with an aeration rate of 1 l. of airl/. of culture/min, measured at 20° and 1·02 atmos. Table 1 shows the mass balance, Table 2 a typical carbon balance and Table 3 the mass balance for reactants and products. The concentrations of bacteria and sorbose, the consumption of oxygen and the production of gaseous CO_2 are shown, plotted against time, in Fig. 1. The oxygen consumed and the CO_2 evolved were calculated from the air flow rate and the O_2 and CO_2 in the effluent air. Specific reaction rates, calculated on the assumption that growth, sorbose formation and oxygen consumption followed

Table 1. *The mass balance for a 3·15 l. batch culture**

Input		Output	
Liquid:		Liquid:	
3150 ml of culture medium (S.G. 1·042) containing (g)		3025·5 ml of crude product (S.G. 1·056) containing (g)	
Sorbitol	308·8	Sorbitol†	0·0
Yeast extract	15·7	Residual yeast extract‡	15·1
Water	2957·8	Water	2882·5
		Sorbose	294·6
		Dried cells	5·0
Gas, containing (g)		Gas, containing (g)	
Atmospheric oxygen**	31·4	CO_2***	6·6
		Water vapour§	108·0
		Unaccounted	1·9
Totals	3313·7		3313·7

* Nett culture time, 15 hr. † Dr. D. Gross (Tate & Lyle, Ltd.) kindly examined samples of fermented culture by paper electrophoresis. No sorbitol was detected.

‡ The organisms would not grow on yeast extract alone or yeast extract and sorbose, but only in the presence of sorbitol. Thus, in making this balance it was assumed that bacterial nitrogen was provided by the yeast extract and bacterial carbon by sorbitol. Dry bacterial substance was assumed to contain 12% nitrogen.

§ Measured by absorbing in activated alumina.

** Calculated assuming there were two reactions: partial oxidation of sorbitol to sorbose and complete oxidation to CO_2 and H_2O.

*** CO_2 in the reactant air was allowed for in deriving this nett figure.

the law of a first order reaction, are given by graph 1 in Fig. 2. Graph 2 in Fig. 2 shows how the quantity $\Delta p/\Delta x$, derived from the data in Fig. 1, changed with time.

Continuous culture. Fig. 3 gives the results for similar operating conditions, with a few observations at 35°: the latter do not differ significantly from those at 30°.

Table 2. *A typical carbon balance for a 3·15 l. batch culture**

Input (g)		Output (g)	
As sorbitol	117·28	As sorbose	113·2
		As bacterial substance†	2·47
		As CO_2	1·39
		Unaccounted	0·22
Totals	117·28		117·28

* These data differ from those in Tables 1 and 3. They were obtained in a separate experiment to check the assumption that oxygen was consumed in two reactions—see note **, Table 1.

† Dry bacterial substance assumed to contain 50% carbon.

Table 3. *The mass balance for reactants and products for a 3·15 l. batch culture*

Reactants (g)				Products (g)		
Sorbitol for	sorbose	297·9		Sorbose		294·6
	CO_2	4·55		CO_2		6·6
	bacterial substance	6·35		Bacterial substance		5·0
			308·8	Water*		
				Source A	29·5	
Oxygen for	sorbose	26·2		Source B	2·2	
	CO_2	5·2				32·7
			31·4	Unaccounted		1·3
Totals			340·2			340·2

* Source A, from oxidation of sorbitol to sorbose. Source B, from complete oxidation of sorbitol to H_2O and CO_2.

Discussion

Relation between growth and product formation in batch culture. It is evident from Fig. 2, graph 2, that the simple concept that the quantity $(\Delta p/\Delta x)$ is a constant was by no means adequate, as during growth this ratio rose from 30 to 120 and then declined. The mean value is about 60.

Sorbitol the limiting substrate in batch culture. From a cursory examination of Fig. 1 it might be deduced that after the seventh hour of growth, sorbose formation and oxygen consumption followed the law of a first order reaction until the culture was substantially complete and that sorbitol was therefore the growth rate limiting substrate. However the more critical test which can be made from Fig. 2 opposes this conclusion. Fig. 2, graph 1, gives the specific reaction rates at time t calculated for bacterial growth, sorbose formation and oxygen consumption, assuming first order processes. Theoretically, at any given time all three values should be the same, and they should have increased in value throughout the lag phase of growth,

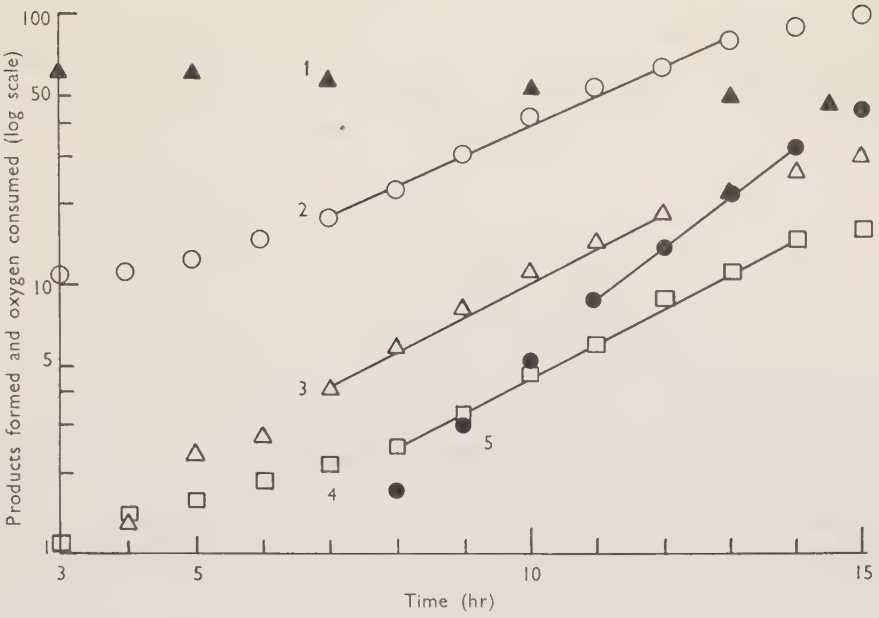

Fig. 1. Batch culture at 30°. The formation of products and the consumption of oxygen as functions of time.

Graph no.	Symbol	Ordinate scale multiplication factor	Information	Average values over the interval a (hr)–b (hr)		
				a–b	μ*	t_d (min)
1	Full triangles	0·1	pH value	—	—	—
2	Open circles	1·0	Sorbose concentration (p, g/l.)	8–13	0·26	161
3	Open triangles	10·0	Cumulative oxygen consumption† (o, mM/l.)	7–12	0·30	141
4	Open squares	0·1	Bacterial dry wt (x, g/l.)	8–14	0·30	141
5	Full circles	1·0	Cumulative CO_2 in exit air† (c, mM/l.)	11–14	0·44‡	96

* $\mu = \frac{\log_e y_{(t=b)} - \log_e y_{(t=a)}}{b-a}$ hr^{-1}, where $y = p$, o, x or c.

† Referred to one litre of culture.

‡ This value, which is significantly greater than the others, is probably false and no doubt due to retention of CO_2 in the culture medium in the form of bicarbonate or carbonate when the pH value is high, and its conversion to dissolved CO_2 and removal as the pH value falls.

remained constant during the exponential phase and fallen off sharply at the end of the culture, as the sorbitol became exhausted. While at any given time the values may not differ statistically among themselves, and while an increase in value occurred during the lag phase, during the so-called exponential phase as deduced from Fig. 1 there was a gradual and statistically significant decrease in specific reaction rate

until the end of the culture. Thus the only rigorous conclusions seem to be that under these conditions product formation is not strictly associated with growth, and sorbitol is not strictly the growth rate limiting factor, since strictly exponential growth was never sustained.

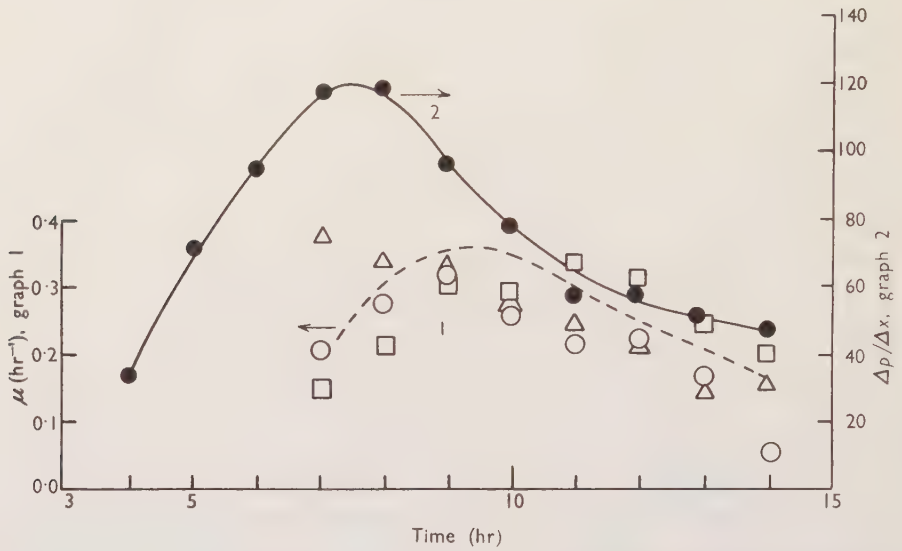

Fig. 2. Batch culture at 30°. Changes with time in the estimated specific reaction rate and the ratio of yield constants.

Graph 1, open symbols, broken line, shows how estimates of the value of the specific reaction rate (μ) of the process changed with time. The data for bacterial growth (x), sorbose formation (p) and cumulative oxygen consumption (o) have been used in the general formula

$\mu_y = \frac{d(\log_e y)}{dt} = \frac{\log_e y_{(t+1)} - \log_e y_{(t-1)}}{2}$ hr^{-1}, substituting x, p and o for y.

Open squares, μ_x; open circles, μ_p; open triangles, μ_o.

Graph 2, full circles, continuous line, was calculated from the results in Fig. 1, using the formula $\Delta p/\Delta x = [p_{(t+1)} - p_{(t-1)}] \div [x_{(t+1)} - x_{(t-1)}]$. It shows how estimates of the dimensionless quantity ($\Delta p/\Delta x$) changed with time.

Continuous culture. The results, shown in Fig. 3, follow the general pattern of a continuous culture. The ratio ($\bar{p}/\bar{x}$), which corresponds to ($\Delta p/\Delta x$) in batch culture, shows some decrease as the dilution rate was increased and its mean value is about the same as that of the batch culture.

There is a striking difference between these results and those for batch culture in the values of the maximum growth rate constant (μ_m). In batch culture the maximum value was not more than 0·35 hr^{-1}, as shown in Fig. 2, graph 1; but in continuous culture output was at a peak at a dilution rate of 0·42 hr^{-1} (Fig. 3, graph 2), so that μ_m must have been greater than this. It was in fact shown to lie between this value and about 0·5 hr^{-1}, at which value the bacterial concentration fell to zero. Another difference, undesirable in the event of industrial application, is that the yield was significantly less in continuous culture (Table 4). Yield might be improved by increasing aeration capacity, by adding a second culture stage, or by re-cycling the

mother liquor from the subsequent crystallizing stage. We did show that if product from continuous culture was shaken in an aerated flask for 48 hr, the sorbose content was raised from an original value of 77 g/l. to 89 g/l., showing that the low yield was not due to a side reaction involving the destruction of sorbitol.

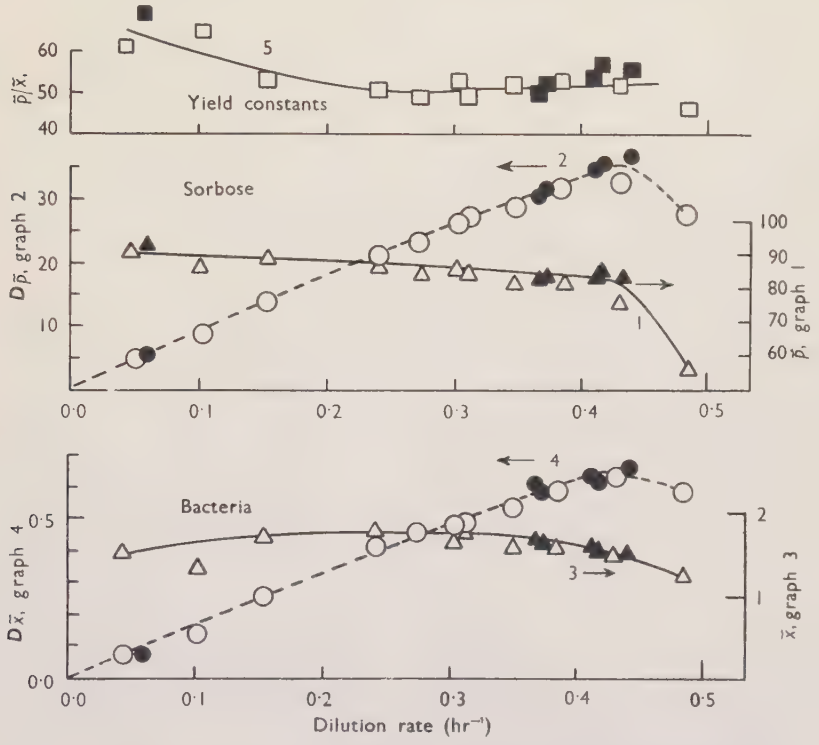

Fig. 3. Continuous culture. The relation between dilution rate and sorbose concentration, $\bar{p}$ in g/l., graph 1, triangles; sorbose production rate, $D\bar{p}$ in g/(l.)(hr), graph 2, circles; bacterial concentration, $\bar{x}$ in g/l., graph 3, triangles; bacterial production rate, $D\bar{x}$ in g/(l.)(hr), graph 4, circles; ratio of yield constants, $\bar{p}/\bar{x}$, dimensionless, graph 5, squares. Open symbols, culture temperature 30°; full symbols, 35°. Maximum output occurs when $D = 0{\cdot}42$ hr^{-1}.

Ratio of the output of the two processes. The results show that the point of maximum output in a continuous culture may not represent optimum production conditions, and in the subsequent cost comparison two cases are considered. The first is where output is at a maximum and the culture vessel size is at a minimum, but where yield is less than maximum. Hence there will be a charge against the product for unconverted sorbitol. The second is where the yield is improved, but as a result production rate is not at a maximum. Here the charge due to unconverted sorbitol will be reduced, but there will be an increased charge due to the larger culture vessel which will be required to produce the same output as in the first case. From graph 2 in Fig. 3 it will be seen that production was at a maximum when the dilution rate ($\boldsymbol{D}$) was 0·42 hr^{-1}. Output was then 35·22 g/(l.)(hr) and the yield (sorbose:sorbitol) 85·6%.

The second case selected is when D was $0{\cdot}21 hr^{-1}$; output was then 18·34 g/(l.)(hr) and the yield 89·2%. Mass balances for these conditions are given in Table 4.

Table 4. *Basic assumptions for a study of the merits of continuous vs batch production of sorbose*

	Batch production	Continuous production	
		Case 1, maximum output, low yield	Case 2, lower output, improved yield
POSTULATES			
Dilution rate, hr^{-1}	—	0·42	0·21
Concentration of sorbose, g/l.	—	83·9	87·4
Output of sorbose, g/(l.)(hr)	5·2*	35·22	18·34
Yield, %	95·4	85·6	89·2
CALCULATIONS FROM ABOVE FOR SORBOSE OUTPUT OF 460 KG/DAY			
Culture vessel capacity, l.	3686	544	1045
Consumption of:			
Sorbitol crystals (98%), kg/day	492·0	548·4	526·2
Yeast extract, kg/day	24·6	27·4	26·3
Medium, l./day	4920	5484	5262
Oxygen consumption, mM/(l.)(hr)	45†	113‡	59‡
ESTIMATES BASED ON FOREGOING			
Cost of culture vessel, £	2500	1000	1700
Amount of fixed capital, £	25,035	15,350	16,080
Operating cost, man-days/day	7 (2 men/shift and 1 man on days)	3 (1 man/shift)	3 (1 man/shift)

* An average rate, taking into account time for harvest and seeding.

† The maximum rate, occurring at the end of the batch.

‡ 116% of the theoretical requirement.

The output of sorbose from the batch vessel (Table 1) was 294·6 g in a nett operating time of 15 hr. But supposing it takes 3 hr to start the next culture, so that the gross operating time is 18 hr, the average output rate from a vessel of 3·15 l. capacity (this includes the seed) will then be $294{\cdot}6/(18\times3{\cdot}15)=5{\cdot}20$ g/(l.)(hr). As the starting quantity of pure sorbitol was 308·8 g, the yield was 95·4% (see Table 4). The ratios of output (continuous culture: batch culture) are thus 6·8 and 3·5, respectively.

Conclusion. While the experiments have been successful in showing that the rate of sorbose production can be enhanced by using the continuous instead of the batch process, the yields in the former are significantly less than those in the latter. Nevertheless, although the lower yields present a technical challenge and scope for further work, the results provide a basis for an examination of the factors which determine profitability. This is attempted in the next part of the paper.

PART II. A COST COMPARISON

Specification of Plant

A production rate of 154 tons/yr in 340 working days (460 kg/day or 19·2 kg/hr) has been selected for comparing the two processes, because it would meet the estimated requirements of the United Kingdom market.

The work taken into account in each process is the preparation of the medium

(dissolve sorbitol and yeast extract in mains water) and its sterilization, the growth of the culture and its storage for the subsequent clarification and crystallization stages. A detailed specification and cost estimate has been made and is summarized below. The basic assumptions are given in Table 4 and the principal components required in the plant are given in Table 5. It should be noted that in calculating the size of the culture vessel it is implicit that the rates of oxygen solution which obtained in the 3 l. vessel shall also be achieved in the much larger vessels which form the hypothetical plant. Estimates for oxygen solution rates are given in Table 4, and we are of the opinion that these values could be attained in vessels of the size considered. This might mean growth under pressure (the culture vessels are assumed to be suitable for operations at 1·5 atmos. gauge), and if this were necessary any subsequent conclusions we have drawn would not be invalidated; but there would be marginal increases in the air costs in the three cases.

Table 5. *The principal items of equipment required for batch and continuous production of sorbose*

Type of equipment	Number and size of items required in		
	Batch culture	Continuous culture	
		Case 1	Case 2
Mixing and feed tanks	nil	2 × 1000 litres	As case 1
Mixing tanks-cum-sterilizers	1 × 185 litres 1 × 3700 litres	A continuous sterilizer and 1 × 1000 litre holding vessel for sterile medium	As case 1
Culture stages	1 × 9 litres 1 × 185 litres 1 × 3686 litres	1 × 30 litres 1 × 544 litres	1 × 50 litres 1 × 1045 litres
Culture storage	1 × 3700 litres	1 × 1000 litres	As case 1
Other equipment	nil	Flow controller and metering device for sterile feed	As case 1

As shown in Table 5, batch culture is envisaged as a three stage process, consisting (apart from shake flasks) of three successive stirred culture vessels of 9 l., 185 l. and 3686 l. capacity respectively. The equipment also includes two vessels used for medium preparation and sterilization, and a culture storage tank equal to the capacity of the culture vessel. The continuous culture plant is assumed to consist of two mixing and feed tanks in parallel, which deliver through a continuous sterilizer into a holding vessel for sterile medium. The sterile medium is then transferred through a flow controller into the culture vessel from where it overflows into a storage vessel which is smaller than in the batch process. In the first case the culture vessel is of 544 l. capacity, and in the second case yield is increased at the extra expense of using a 1045 l. culture vessel and a larger batch seed vessel.

Cost of Fermentation Stage

A summary of the cost estimate is given in Table 6. This shows that for continuous culture, where production rate is at a maximum but the yield is lower (case 1), the

resulting excess cost of sorbitol and yeast extract exceeds the remaining savings; relative to batch production sorbose will cost, in this case, an extra 1·30d/kg. If, however, as in case 2, the dilution rate is reduced by increasing the culture vessel size (which incidentally makes a less rigorous demand on the aeration system), then the yield is increased, the cost of raw material reduced and the difference between the continuous and batch processes is now 3·59d/kg of sorbose in favour of the continuous process. It will be seen that if the yield in continuous culture matches that of the batch method, the potential maximum saving amounts to almost 12d/kg of sorbose, achieved principally by economy of labour.

Table 6. *Unit costs (pence) for continuous and batch production of sorbose*

Nature of expenditure	Cost (d/kg) under			Excess of costs for batch over continuous culture	
	Batch culture	Continuous culture			
		Case 1, maximum output but low yield	Case 2, improved yield	Case 1	Case 2
Direct manufacturing costs excluding raw materials					
Steam and cooling water	0·80	0·89	0·85	−0·09	−0·05
Electricity for agitation and air supply	1·82	0·47	0·74	+1·35	+1·08
Operating staff	5·50	2·40	2·40	+3·10	+3·10
Indirect manufacturing costs					
Overheads on operating staff	11·00	4·80	4·80	+6·20	+6·20
Fixed manufacturing costs					
7%/yr plant depreciation	2·69	1·65	1·73	+1·04	+0·96
Sub-totals	21·81	10·21	10·52	11·60	11·29
Raw materials					
Sorbitol	86·00	95·00	91·20	−9·00	−5·20
Yeast extract	35·30	39·20	37·80	−3·90	−2·50
Grand totals	143·11	144·41	139·52	−1·30	+3·59

Discussion

The assertion that it should be possible to produce sorbose by continuous methods at a cost ranging from 4d/kg to 12d/kg cheaper than by batch methods gives no datum line for comparison, and it is better to compare the estimated return on the capital invested in each process (Aries & Newton, 1955). On an output of 154 tons of sorbose/yr a saving of 3·59d/kg (case 2) amounts to £2340 each year, which is available as extra profit. Let the gross profit which is taken to render the batch process a profitable concern be equal to £P, which sum is distributed over £25,000 of fixed capital. If, however, the sorbose is made by the continuous method and sold at the same price the revised profit figure is £(P+2340), to be distributed this time over some £16,000. It remains to arrive at a value for P in order to compare the profitability of the processes. Chambers, deputy chairman of I.C.I. Ltd., said in 1957 that 'A project which yields less than about 12½% on the capital employed would . . .

be regarded as wholly unattractive and barely able to pay a reasonable dividend without any margin for reserves'. Taking this value, and solving the equation $P/250=12\frac{1}{2}$, gives P=£3125. Thus the potential gross profit from the continuous process is £3125+£2,340=£5465. This is distributed over £16.000 of fixed capital instead of £25,000 as formerly, giving a yield of 34%, as opposed to $12\frac{1}{2}$% for the batch process. This example does not necessarily represent the operating point at which unit cost is a minimum and profitability at a maximum. We have not attempted to solve that problem, since in the example chosen the discrepancy between 34% and $12\frac{1}{2}$% shows that continuous culture would be a profitable venture. A similar result can be expected for other examples of continuous culture, each of which would have to be examined on its own merits to discover the effect of change in direct, indirect and fixed manufacturing costs resulting from the change-over from batch to continuous culture.

There has been adverse criticism of the industrial possibilities of continuous culture (Microbiology Group Meeting, 1957) and it is hoped that these speculations will provoke comments from workers directly interested in the cost aspects of biosyntheses.

The problem of assessing the profitability of the continuous process was set us by Dr. D. W. W. Henderson, and we are grateful for his constructive criticism. We also thank Messrs. E. Kirk and D. Lamb of the Ministry of Supply, Directorate of Ordnance Factories, and Mr. P. R. Sprigings of Commercial Solvents (Great Britain Ltd.), for facts on plant costs. We are grateful to Mr. J. J. H. Hastings, M.B.E., of The Distillers Co. (Biochemicals) Ltd., for criticism of the manuscript and Mr. S. Peto (statistician) for help in analysing and presenting the results. The work was started in conjunction with a former colleague, Mr. V. Williams, now of Albion Distilleries Ltd., Vitulugt, British Guiana.

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THE VALUE OF THE ASSOCIATION ESCHERICHIEAE–GROUP D STREPTOCOCCI IN THE DIAGNOSIS OF CONTAMINATION IN FOODS

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SUMMARY: The tests for faecal contamination in foods, based on 'indicator species', should be reconsidered for the following reasons. First, in Europe, *Escherichia coli* is regarded as a specific index of such contamination; but, apparently through the use of antibiotics, the proportion of human faeces containing *Klebsiella* has risen from 5·2% in 1947 to 48·4% in 1956–7. Moreover, *E. coli* and other coli-aerogenes organisms may be absent from the gut of certain animals, notably the pig. Second, while the usual methods of counting coli-aerogenes bacteria do not permit the isolation of strains which ferment lactose slowly or not at all (i.e. paracolons), these occur in 35·6% of samples of human faeces. Further, the recommended methods of detecting such strains are not wholly satisfactory. Third, the origin of the coli-aerogenes bacteria is uncertain.

It is thus necessary to consider other organisms which normally inhabit the intestinal tract of humans and other animals. As to *Clostridium perfringens*, its source is doubtful, for it may come from faeces or from soil, and many foods contain only soil strains. The group D streptococci, on the other hand, are excellent indicators of faecal contamination. They are constant or frequent in the intestines of man and animals, and often more numerous there than coli-aerogenes bacteria. Their specificity as an index of faecal contamination is high and their investigation is easy with the selective media now available. Associated with the coli-aerogenes bacteria, they justify the diagnosis of faecal contamination in a foodstuff.

Under the influence of new conditions of living and feeding, the faecal flora of humans and animals may be altered. Our notions about the means of assessing the faecal contamination of foodstuffs may therefore have to be modified, in the light of new ideas derived from general bacteriology, and of better techniques. In many countries, the coli-aerogenes bacteria are still regarded as the best indicators of faecal contamination: but in Europe, for a long time past, particular importance in this regard has been attached to *Escherichia coli*. These two notions are now open to criticism.

The value of tests for coli-aerogenes bacteria

Ten years ago, *E. coli* was the most frequent and abundant of the *Escherichieae* in human faeces. However, the use of antibiotics active against *E. coli* (e.g. chloramphenicol, tetracyclines) has greatly modified this situation (Table 1), so that in 1956–7 *E. coli* was found only in 86·1% of cases, while *Klebsiella* strains, which are much more resistant to the antibiotics, were present in 48·4%. Often, in such cases, the latter were so strongly preponderant that the ratio *Klebsiella*:*E. coli* exceeded 100:1.

Table 1. *Changes in the incidence of certain coli-aerogenes bacteria in human faeces*

Authors	Year	No. of samples	Occurrence (% of samples) of		
			E. coli	*Cit. freundii*	*K. aerogenes*
Skinner & Brudnoy	1932	585	—	—	0·5
Buttiaux	1947	1000	94·2	4·8	5·2
Buttiaux	1956–7	4461	86·1	9·8	48·4
Njoku-Obi & Skinner	1957	—	—	—	27

Table 2. *The incidence of certain coli-aerogenes bacteria, including paracolon strains, in* 1956–7, *in* 4461 *samples of human faeces*

Organism	Incidence (% of samples positive)
E. coli	86·1
Parac. coliforme	15·4
Cit. freundii	9·8
Bethesda (*Citrobacter*)	3·5
Parac. intermedium	1·2
K. aerogenes	48·4
Parac. aerogenoides	15·5
Total of slow fermenters	35·6%

The techniques normally in use reveal those *Escherichieae* which ferment lactose rapidly; but they give little information about strains which do not ferment lactose or do so only slowly, strains generally called *Paracolobactrum* or '*B. paracoli*'. These, however, are frequently present in human faeces, where we have found them in 35·6% of cases (Table 2).

It has been suggested, by Henriksen (1955*a*) for water and by Mossel (1957) for foods, that this deficiency might be overcome if the media with lactose were replaced by media with mannitol. This proposal seems unsound, at present; for there occur, in water and in certain foodstuffs, mannitol-fermenting bacteria which are never encountered in the faeces of warm blooded animals. This is particularly true of species of *Aeromonas*. These organisms play an important part in the biological processes on which the manufacture of raw sausage ('saucisson cru') is based and, if the proposed technique were followed, it would lead to the systematic rejection of products which are perfectly wholesome (Table 3). With water it is the same: *Aeromonas* is found in surface waters free from faecal contamination. In oysters and other molluscs, devoid of coli-aerogenes organisms, there are strains of *Beneckea* which, despite the statements in the 7th edition of Bergey's Manual (Breed, Murray & Smith, 1957), attack mannitol rapidly. It is important to realise the difficulty of identifying *Aeromonas* strains promptly; their chief biochemical properties would usually cause them to be regarded as *K. cloacae* or '*B. paracoli aerogenoides*' (*Hafnia*), from which they can be distinguished solely by their polar flagella (Plate 1).

It is only in comparatively recent years that there have been thorough investigations, with well tried techniques, of the faecal flora of warm blooded animals; though these, as much or more than man, may be the reservoirs of dangerous intestinal pathogens.

Hence it has only recently become plain that the distribution of *E. coli* and other coli-aerogenes organisms is not the same in their intestinal floras as in that of humans. These organisms, which are present and abundant in the intestinal tract of the cow, for example, are always rare in that of the pig and sometimes absent (10·6% of cases).

Table 3. *The numbers of coli-aerogenes bacteria and* Aeromonas *in raw cured sausage ('saucisson cru')*

Age of sausage* (days)	Numbers (10^4/g) of	
	Coli-aerogenes bacteria	*Aeromonas*
1	3·0	8·0
3	2·5	25
7	1·0	4·2
15	None	2·0
22	None	1·2

* This sausage is incubated for 7 days at 20–25°, then it is placed in a drier for 8 days at 16–18°, and finally it is kept at room temperature (about 18°) for 15 days. The time is reckoned from the commencement of incubation at 20–25°.

Even if a certain and rapid technique were found for detecting the presence in foods of *Escherichieae* with all the possible lactose reactions (positive, slow positive and negative), the results obtained would still be difficult to interpret. It has been shown that *E. coli* is not always an index of faecal contamination in citrus juices (Wolford, 1956); and conversely, one finds many strains of *Citrobacter freundii*, *K. aerogenes* and especially *K. cloacae* in foods which have not undergone faecal pollution. Henriksen (1955*b*) made a suggestion which might be useful, and which we have verified in Northern France (Buttiaux, Samaille & Pierens, 1956), namely that *Klebsiella* strains of faecal origin possess a urease, readily demonstrable at 30°, which does not exist in the others; but this phenomenon needs more extensive study before it can be regarded as fully confirmed.

Eddy & Kitchell (1959) have just raised doubts about the origin of Gram-negative bacilli which ferment lactose slowly. Many of the organisms of this type which they isolated from chilled meats were psychrophiles. Hence, one might think that they would not be able to live in warm blooded animals: but this would, however, be debatable, for we have isolated psychrophilic Enterobacteriaceae from human faeces: these were strains of *Providencia* and *Proteus hauseri*.

The value of tests for other indicator organisms

It is undeniable that the above considerations greatly diminish the value of 'colimetry' for detecting faecal contamination of foodstuffs. It is thus necessary to consider the use of other indicator species which are commonly found in the faecal matter of man and other animals, i.e. *Clostridium perfringens* and the faecal streptococci.

Cl. perfringens. This can be rejected at once. Beerens & Delcourte (1958) have just shown that it is possible to distinguish between strains from faeces and those from soil. Following their procedures, it has been recognized that the presence of faecal strains is exceptional in heavily contaminated products like gelatin, where soil strains occur almost exclusively.

The faecal streptococci. These, in contrast to *Cl. perfringens*, merit consideration, though it must be emphasized at the outset that, for the objects here in view, the concept of 'enterococci' must be set aside. In addition to *Strep. faecalis* and its varieties *zymogenes* and *liquefaciens*, and *Strep. faecium* and *Strep. durans*, the organisms to be considered must include *Strep. bovis*, which, as Shattock (1949) has shown, belongs to group D, because it occurs frequently in the gut of animals. Also, if possible in the future, *Strep. equinus* ought to be included, and the need to do this draws attention to the fact that the media hitherto recommended for the investigation of enterococci would certainly not be suitable for all the faecal streptococci.

The faecal streptococci, in this sense, are common in the intestinal tract of humans of all ages, and in that of the pig, and they are frequent in that of other domestic animals. They are often more numerous than the *Escherichieae* in the gut of such animals (Table 4). Their sensitivity as indicators of faecal contamination is about the same as that attributed to *E. coli*; this has been verified with waters drawn from 106 different catchments known to be subject to slight but intermittent faecal pollution. Using *E. coli*, pollution was detected in 92·4% of the waters, while using group D streptococci the figure was 90·5%. They are of good specificity; in 10% of waters where on examination the only faecal indicator organism found at first was a group D *Streptococcus*: the others appeared later. *Cl. perfringens* gave only 74·5%.

Table 4. *The frequency of occurrence and the relative numerical proportions of group D streptococci and coli-aerogenes bacteria in human and animal faeces*

Type of faeces	Occurrence (% of samples positive) of		Ratio of numbers Group D:coli-aerogenes
	Coli-aerogenes bacteria	Group D streptococci	
Human	86·1	100·0	0·6 (1)*
Pig	89·4	100·0	21·0 (2)
Cow	100·0	65·6	6·4 (1)
Sheep	98·0	87·5	0·7 (1,2)

* (1) Data from Slanetz & Barthley (1957); (2) from Buttiaux (1958).

Most of the species or strains of faecal streptococci (except *Strep. bovis*) survive better than the coli-aerogenes bacteria in natural media (e.g. water) or in products which have more or less well recognized inhibitory properties for the latter. Appleman (1955) has shown this with citrus juices, Zaborowski, Huber & Rayman (1958) for frozen foods, and we ourselves for meat brines (Buttiaux & Moriamez, 1958). In all such cases, therefore, these streptococci afford the more sensitive test for revealing faecal contamination. They can be investigated with ease and certainty

by employing the excellent media now available, such as that of Barnes (1956) which is preferred for foods, that of Litsky, Mallman & Fifield (1953) which gives good results with water, and that of Slanetz & Barthley (1957) which is adaptable to the membrane filter technique (for discussion see Barnes, 1959).

These considerations prove the indisputable value of the faecal streptococci as indicators of faecal pollution, which numerous workers have amply confirmed. In France, their investigation has been recommended since 1949 in the interpretation of the sanitary quality of products containing coli-aerogenes bacteria other than *E. coli*.

Conclusion

This discussion leads to the conclusion that, in view of the uncertainties of 'colimetry' and the difficulties in interpreting the results correctly, it is always essential, when judging the sanitary quality of a foodstuff, to look for faecal streptococci also. In our view, the simultaneous presence of a member of that group and a coli-aerogenes strain justifies the verdict that there has been faecal contamination. In the investigations so far made on this subject, no exception to this principle has been recorded.

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Explanation of Plate

Electron micrographs (gold shaded) of organisms taken from cultures in sloppy (0·5%) agar to show contrasted flagellation of (a) *Aeromonas* from raw sausage–polar (×10,000) and (b) *E. coli*–peritrichous (×15,000).

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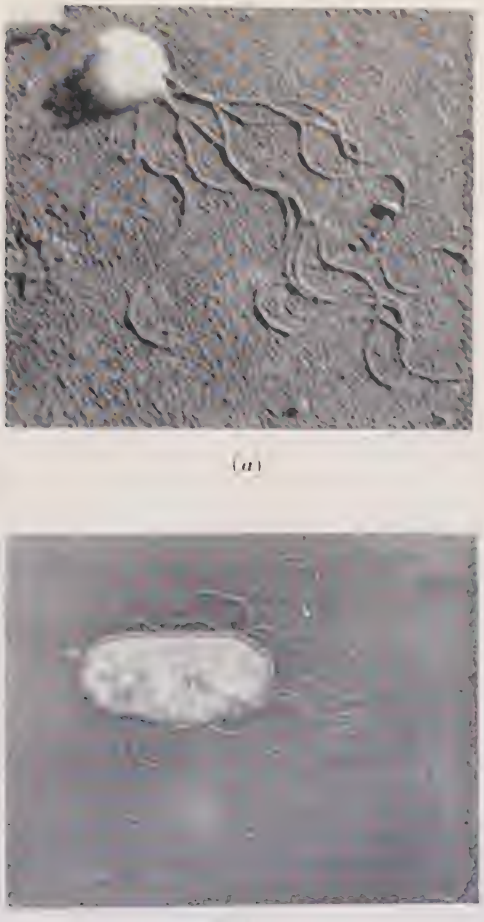

(*a*)

(*b*)

R. Buttiaux — The Value of the Association Escherichieae–Group D Streptococci in the Diagnosis of Contamination in Foods. Plate 1

Henry, P. S. H. (1959). *J. appl. Bact.* **22** (1), 159–173.

PHYSICAL ASPECTS OF STERILIZING COTTON ARTICLES BY STEAM

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SUMMARY: In this paper some of the physical processes occurring when cotton articles are sterilized in metal drums or bare packages in an autoclave are discussed, with special reference to the case where a good vacuum is drawn before the admission of steam. It is shown that under these conditions the heating will be quick, uniform, regular and reversible, and that no separate drying-off period should be needed. Calculation of the temperatures and moisture contents reached during the 'quasi-equilibrium' period shows that the 'superheating' of the cotton is self-limiting, can never exceed a few degrees Centigrade, and will not give rise to serious weakening of the cotton. Calculation of the rate of irreversible penetration of moisture into the contents of the drum shows that this can easily be limited to the wrapping layer. Information is given on the 'heat-tendering' of cotton, and the advantages of using a good vacuum are summarized.

The following discussion of some of the physical processes occurring when dressings and other cotton materials are sterilized in drums or packages within an autoclave was originally intended as an appendix to the experimental paper by Penikett, Rowe & Robson (1958) but, after a delay in its writing, it has now been made into a separate paper. In this subject, as always, it is the experimental evidence which is the final arbiter, and the following theoretical discussion (based wherever possible, however, on experimental data) is intended partly as a guide for further experiment and partly to explain certain things which may be unfamiliar, or even surprising, to those who have not previously studied the heating of textiles by steam. It is hoped, also, that the evidence presented may allay the fears of those who have suggested that the use of a good vacuum before admission of steam might damage the cotton. Some of the predictions made here have subsequently been verified experimentally: the reversibility of the moisture uptake (Penikett, Rowe & Robson, 1958) and the approximate magnitude and long duration of the 'superheating' (Penikett, pers. comm.).

Physical Processes Occurring During Sterilization

Cotton and similar cellulosic materials can absorb as much as 20% of moisture, calculated on the dry weight, or in some cases even more, without any free liquid being present on the surface of, or between, the fibres. This is not in contradiction with the observation of Penikett, Rowe & Robson (1958) that towels begin to feel damp when they contain as little as 6% of moisture, for this early damp feeling is due to the increased thermal capacity of the cotton containing moisture, and to the greater flexibility of the moist fibres, which allows closer contact with the skin; both changes increase the cooling effect on the latter. The amount of moisture absorbed at equilibrium is determined mainly by the relative humidity of the surroundings,

with a comparatively small diminution of moisture content as the temperature rises at constant relative humidity (Urquhart & Williams, 1924). The moisture is distributed through the substance of the fibres, and is held by physical bonds of varying degrees of intensity. A result of this close association between the cellulose and the water is that an appreciable amount of heat is given out when liquid water is added to dry cotton, an effect which is partly responsible for the warm feeling of cotton (or woollen) materials taken straight from the drying cupboard. It is the first 1 or 2% of moisture added from the dry state that is responsible for most of the heat evolved, subsequent additions giving out much smaller amounts. If, however, the moisture comes directly from the atmosphere, or from dry steam, the ordinary latent heat of condensation of steam will be given out in addition to the heat of wetting, and will, except for cotton starting quite dry, form the major part of the heat evolution. It is this heat of condensation and wetting, often spoken of together as the 'heat of absorption', aided at first by the mere fact that the steam is hot, which heats up the cotton dressings in the sterilizer. Provided that most of the air has first been evacuated from the vessel and from the pores of the dressings, the steam penetrates the latter quickly and completely, and evolution of the heat of absorption raises the temperature of the whole much more quickly and uniformly than could be achieved by any dry heating process. The dressings rise first to the temperature of the steam, after which they cannot receive heat merely by conduction from the steam; they continue, however, to rise in temperature owing to the fact that the cotton has not yet received as much moisture as it can take up from saturated steam, and therefore the evolution of heat of absorption can continue. The dressings do, in fact, become hotter than the steam; but as soon as they do so the relative humidity of the steam in immediate contact with them (i.e. that contained within the package) becomes less than 100% in the ratio of the actual pressure of the steam to the saturated steam pressure at the temperature of the dressing. Now we have seen that the amount of moisture which a textile contains when at equilibrium with its surroundings depends mainly upon the relative humidity; hence the ability of the dressings to absorb further moisture (and therefore to continue to rise in temperature) rapidly diminishes as their temperature exceeds that of the steam, and there soon comes a point, only a few degrees above the steam temperature, where a temporary state of 'quasi-equilibrium' is reached and the temperature ceases to rise. Thus the 'superheating' of the dressings is a self-limiting process.

Up to now the chief processes have been reversible in the thermodynamic sense. Subsequently, if the dressings are left long enough in the autoclave, they will slowly lose heat to the steam by conduction, while gaining further moisture, until the final, true equilibrium is reached at the temperature of the steam and a moisture content of the order of 20%. This second stage in the process is irreversible, being controlled by the rate at which heat can pass by conduction from the package, but it will be shown below that for drums or textile packages full of dressings this conduction will proceed so slowly that it can be neglected in practice, though not necessarily for smaller objects. The rate at which the cotton absorbs further moisture cannot now exceed that at which all the heat liberated is lost by conduction to the steam, owing to the limiting process mentioned above; consequently in practice, with drums

full of dressings, both temperature and moisture content remain virtually stationary and uniform throughout most of the interior of the package for a long time (many hours at the centre). The way in which the temperature and moisture content of the cotton vary with time is shown diagrammatically in Fig. 1 where, however, the rate of approach to final equilibrium in the second stage has been greatly exaggerated in comparison with the rate of rise in the first stage. Had the two stages been plotted on the same time scale, both curves would have appeared practically horizontal after reaching the 'quasi-equilibrium' stage.

From the calculations given below it is anticipated that, with dry steam at 20 lb/in.^{2} gauge pressure and 126°, the stationary temperature of the cotton would be about 9° above that of the steam for materials quite dry at the start, but only a degree or two for materials initially containing 5% of moisture, the value given by Penikett *et al.* (1958) as common for cotton goods taken from the hospital store. The corresponding temporary moisture contents at the high temperature are expected to be about 6% and 12% respectively. Cotton initially containing 8% of moisture or over would not be expected to rise above the steam temperature at all.

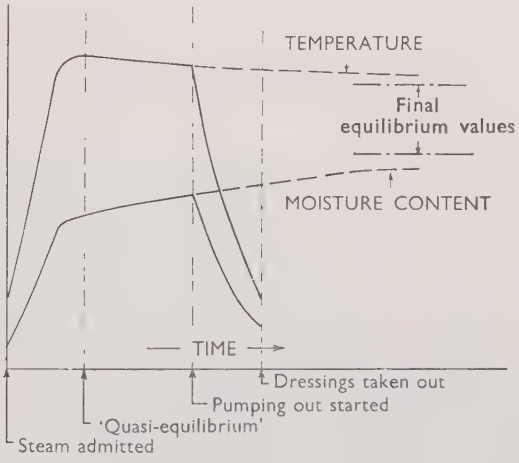

Fig. 1. Diagrammatic representation of the changes with time of the temperature and moisture content of cotton materials heated by steam with initial vacuum, showing the rapid ascent to the 'quasi-equilibrium' state, the very slow subsequent change towards true equilibrium, and the rapid return to the initial state when the steam is pumped out.

Since the conditions of maximum temperature and moderate moisture content (the 'quasi-equilibrium' state) were reached by processes mainly reversible, and since the irreversible processes which then occur are expected to require, for large packages, times much greater than those needed for sterilization, it is deduced that, providing dry steam is used with a good initial vacuum to enable the steam to penetrate the whole package quickly, the dressings should rapidly and simultaneously return to their initial temperature and moisture content when the steam is pumped out. The process here is an exact reversal of the heating-up stage, the dressings being cooled

by the evaporation of their absorbed moisture. We should not, therefore, expect any prolonged drying process to be necessary, the dressings being dry enough as soon as they are cool enough to be taken out. The experimental results of Penikett, Rowe & Robson (1958) showed that this is indeed so. The effect is illustrated by the descending curves in Fig. 1, in which the removal of the dressings has been made to coincide with the time when they reached room temperature, their moisture content then being slightly (probably not more than 1%) greater than when they were put in, on account of the hysteresis in the moisture relations of cotton.

Calculation of Superheating and Temporary Moisture Content

The symbols used below have the following meanings:

m is the dry mass of the cotton, in grammes; M_o and M are the fractional moisture contents at the start, and at any time, respectively, reckoned on the dry weight; t_o and t are the temperatures of the cotton at the start, and at any time, respectively, in °C; t_s is the temperature of the steam, in °C; T is the absolute temperature corresponding to t, in °K; p is the total pressure of the steam, in mm of mercury; W and W_o are the integral heats of wetting of the cotton when the moisture contents are M and M_o, respectively, i.e. the heat given out in cal/g of dry cotton, when 1 g of cotton (measured dry) having the stated moisture content is immersed in an excess of liquid water; L and L_s are the latent heats of evaporation of water at temperatures t and t_s, respectively, in cal/g.

The first step was to plot against temperature the equilibrium moisture content for cotton absorbing at a constant partial pressure of water vapour equal to that of the steam. The data for this were derived from the experimental work of Urquhart & Williams (1924). On the same diagram, and using the same scales, curves were then plotted showing the temperatures which would be reached by a cotton material at the various instantaneous moisture contents as the steam was absorbed by it—one curve for each initial moisture content. These are determined solely by consideration of the heat exchanges. The conditions of 'quasi-equilibrium' are then given by the point at which the equilibrium curve cuts the heat exchange curve for the appropriate initial moisture content. These curves are shown in Fig. 3; their detailed derivation is considered below.

Extrapolation of equilibrium data

Unfortunately, the most comprehensive data for moisture absorption by cotton (Urquhart & Williams, 1924), though they reach nearly to the required temperature, do not go quite far enough for the present purpose and it has been necessary to extrapolate them by means of the Clapeyron-Clausius equation in the integrated form:

$$\log p = F + G/T \qquad (1)$$

where F and G are dependent upon the moisture content M, but are taken to be nearly independent of T over the small range of temperatures concerned. The quantity G should be equal to the heat of absorption from the vapour, divided by the gas constant R, and multiplied by $\log_{10}e$ if logarithms to base 10 are used. No

use, however, was made of this fact, since experimental determinations of the heats of absorption or wetting are not available for the high temperatures concerned, and it seemed safer to determine G from the experimental values for p at different temperatures as near to those concerned as were available. Urquhart & Williams (1924) showed that their results for a given moisture content, when log p was plotted against 1/T, gave lines of slight curvature for which straight lines were a sufficient approximation, especially if different straight lines were used for the results above and below 60°. In the present calculation the slopes and positions of the lines for the results above 60° were used to find F and G for the various moisture contents. F and G were then plotted against M, as shown in Fig. 2.

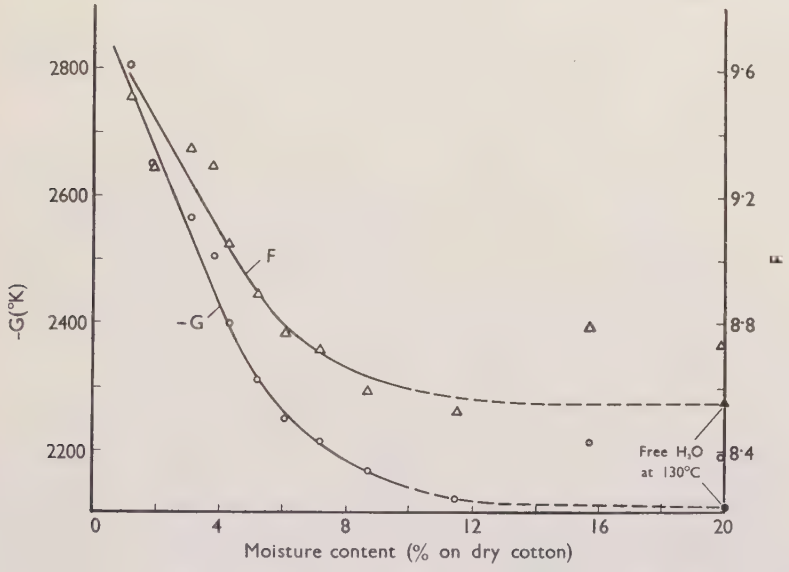

Fig. 2. The relation between the moisture content and the Clapeyron-Clausius coefficients F and G for moisture absorption by cotton (see text).

This is not the place to discuss the curious divergence of the last two points at the lower end of the curve for G from the smooth curve suggested by all the other points. This corresponds to a feature in Urquhart & Williams's results which indicates a negative heat of absorption (from the liquid) at high temperatures and relative humidities, a matter on which there appears to be some difference of opinion. Fortunately, for the present calculations only the lower moisture contents are important, and here the extrapolation is reasonably certain, for in this region Urquhart & Williams (1924) carried their observations up to higher temperatures and, indeed, for moisture contents of 2% and below, within a few degrees of the temperatures that are here concerned. Moreover, no question of a negative heat of absorption arises here. The lower end of the curve for G has been made into a smooth curve tending, as it must, towards the value of G for free water at 130°. This end of the

curve will only be required for dressings which start rather damp, and we shall see that the predicted 'superheating' is likely to be negligible under these conditions.

When the curve for G had been drawn, the curve for F was established using values for G derived from the smooth curve.

The moisture equilibrium curve

From the dependence of F and G on M, it was possible to plot the moisture equilibrium curve for the chosen steam pressure by choosing successive values for M, substituting the corresponding values for F and G in equation (1) together with the value for p (1798 mm of Hg, corresponding to 20 lb/in.^{2} gauge pressure), and solving for T. Thus was derived the curve marked 'moisture equilibrium' in Fig. 3. It will be understood that, though this curve gives the value of the moisture content M at which the cotton will be in equilibrium with steam at the given pressure and temperature t, there will not be thermal equilibrium if the cotton at temperature t is surrounded by steam at temperature t_s.

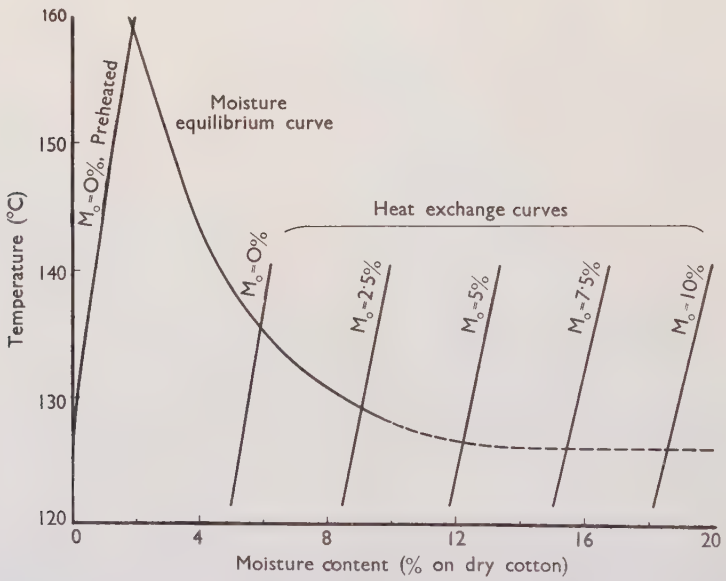

Fig. 3. The 'moisture-equilibrium' curve for cotton in steam at 20 lb/in.^{2} gauge pressure and 126°, showing the temperatures at which the cotton can exist in 'quasi-equilibrium' at varying moisture contents. Also shown are heat-exchange curves giving the instantaneous temperatures reached at various moisture contents as the cotton is heated up by absorption of moisture, one curve for each initial moisture content, and a curve for cotton which started hot and dry.

The above method of producing the moisture equilibrium curve was that finally used. At first, use was made of the modified form of the Clapeyron-Clausius equation suggested by Urquhart & Williams (1924), but this gave a curve with some rather

extraordinary features, and it was thought better to use the usual form of the equation, especially as this made it easier to interpret the constants in terms of simple physical quantities, if necessary.

The heat exchange curves

To calculate these curves, showing the relation between the temperature (t) reached and the amount of moisture absorbed from the steam by the cotton as it heats up, we may imagine the absorbed moisture to have been first condensed at t_s, then cooled (or warmed) to t, and finally absorbed by the cotton, which has been warmed to t. We then equate the heat given out by the former processes to that required by the last.

It will be assumed that the latent heat of condensation (in cal/g) of steam at temperatures between 100° and 150° is given by

$$L = 606 - 0{\cdot}677\ t \tag{2}$$

and that the specific heats of cotton (dry) (0·33 cal $deg^{-1}g^{-1}$) and water (1·00 cal $deg^{-1}g^{-1}$) are constant and additive. This latter assumption is not strictly true, but is nearly enough so for the present purpose, though a better approximation is used later. It involves the assumption that the heat of wetting W is independent of temperature; this also will be assumed. Thus we have

$$\text{Heat evolved} = m(M-M_o)\{606-0{\cdot}677t_s+1{\cdot}00(t_s-t)\}+m(W_o-W) \tag{3}$$

and

$$\text{Heat required} = 0{\cdot}33m(t-t_o)+1{\cdot}00mM_o(t-t_o) \tag{4}$$

Equating these, simplifying, and solving for t in terms of M, we find that

$$t = \frac{(M-M_o)(606+0{\cdot}323t_s)+(0{\cdot}33+M_o)t_o+(W_o-W)}{0{\cdot}33+M} \tag{5}$$

If now we can assume that the dressings will be put in at 20°, and that the steam will be at 20 lb/in.² gauge pressure, we can simplify equation (5) by inserting $t_o=20$ and $t_s=126$ and obtain

$$t = \frac{(6{\cdot}6-627M_o+W_o)+(647M-W)}{0{\cdot}33+M} \tag{6}$$

We can now draw the heat exchange curves (nearly straight lines, see Fig. 3) relating t to M for individual values of the initial moisture content M_o, inserting also in equation (6) the corresponding value for W_o obtained from the experimental papers of Rees (1948) and Guthrie (1949), whose results are reproduced as a graph in Fig. 4. For example, if the dressings start with a moisture content of 5%, we substitute $M_o=·05$ and $W_o=3{\cdot}7$ cal/g, which gives

$$t = \frac{-21{\cdot}0+647M-W}{0{\cdot}33+M} \tag{7}$$

Since W depends upon M, it has to be obtained from the curve in Fig. 4 for each value of M. However, for the last three of the lines in Fig. 3 (towards the upper end of the abscissa) the effect of W on equation (7) is almost negligible, and although the values of W were needed for moisture contents beyond those covered by the direct experimental data, it was considered in order to use an extrapolation based on the similarity in shape of the curves for various textile materials (Guthrie, 1949).

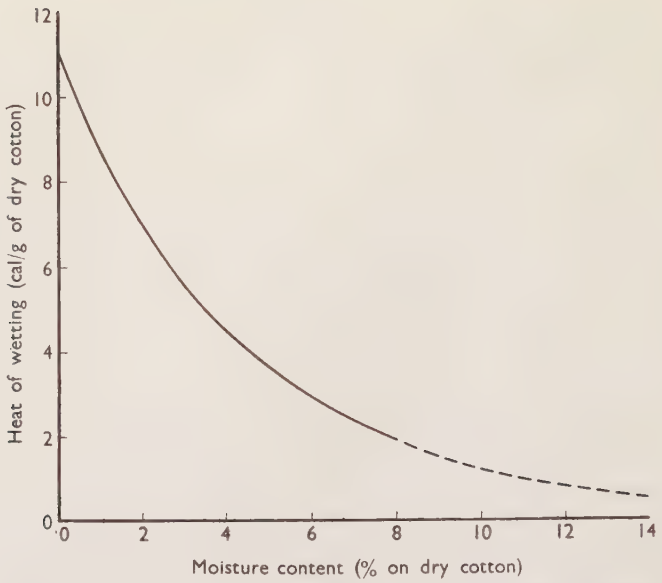

Fig. 4. The relationship between the heat of wetting of cotton and the initial moisture content, derived from the data of Rees (1948) and Guthrie 1949).

The points where the heat exchange curves shown in Fig. 3 for initial moisture contents of 0, $2\frac{1}{2}$, 5, $7\frac{1}{2}$, and 10% cross the moisture equilibrium curve give the conditions of 'quasi-equilibrium'. It will be seen that only if the dressings are put in fairly dry do the curves cross at a position where the path of the moisture equilibrium curve is reasonably certain. Only in this case, then, can the actual value of the 'superheating' be predicted; for the rest, however, this will not be more than a degree or two, and that is all that needs to be known for the present purpose. On the other hand, the nearly vertical slope of the heat exchange curves means that the exact course of the moisture equilibrium curve makes little difference to the predicted moisture contents during the 'quasi-equilibrium', and figures for these are given in Table 1. It will be seen that even when the cotton starts dry the superheating is not expected to exceed 9° at the centre of the package, if this is initially at room temperature; in the next section it will be shown that the superheating is expected to be less near to the surface than at the centre. Both these predictions have been confirmed experimentally by Penikett & Duncan (pers. comm.). When they started with cotton towels containing 1% of moisture, and reduced the air pressure to 20 mm Hg before admitting steam, they obtained an average value of 7·1° for the central superheating and 4 or 5° for that near the surface. With gravity displacement instead of the vacuum, the results were of the same order, though the rate of approach to the maximum temperature was much lower. These figures must be compared with the values of 25° and 50° obtained by Walter (1948) for the superheating of the interior and surface, respectively, of a package of cotton, initially dry (and hot). This great difference in the experimental findings is undoubtedly connected with

the fact that Walter's package had been kept in the autoclave in air at 121° for 10 hr before steam was admitted, so that it had time to become preheated—circumstances which would not occur in practice.

Table 1. *The temperature and moisture content of cotton during the 'quasi-equilibrium' period, for various initial moisture contents, in dry and saturated steam, at 20 lb/in²*

Initial moisture content (%)	Values at 'quasi-equilibrium' of		
	Temperature (°C)	'Superheating' (°C)	Moisture content (%)
0	135	9	6
2½	129 (?)	3 (?)	9
5	Close	Small,	12
7½	to	probably	15
10	126	negligible	18

The expected superheating for preheated dry cotton can be obtained by putting $t_o=126°$ and $M_o=0$ in equation (5); this gives the expression

$$t=\frac{52{\cdot}8+565M-W}{0{\cdot}33+M} \qquad (7.1)$$

from which the line in Fig. 3 marked 'Preheated' was derived. The predicted 'quasi-equilibrium' temperature is 159°, representing a superheating of 33°. This, therefore, goes part of the way towards explaining Walter's observations. In the next section it will be shown that neither preheating nor drying would be expected to affect more than a very thin layer below the surface during the few minutes required in practice to draw a good vacuum—a conclusion which again has been verified experimentally by Penikett & Duncan (pers. comm.).

Calculation of the Rate of the Irreversible Stage

When the cotton has (by rapid reversible processes) reached its state of 'quasi-equilibrium', in which it is a little hotter than the steam and in equilibrium with it as far as moisture is concerned but not as regards temperature, any further change will take place by irreversible processes involving conduction of heat and diffusion of moisture. The cotton will continue to gain moisture, but only as fast as its rate of loss of heat to the steam will allow. An estimate of the rate at which this will occur is required, in order to predict whether these irreversible processes will be important. It is shown below that the penetration into the package of extra moisture arising thus should be so slow that in practice only a thin outer layer would be affected; this is usually only a wrapping.

The effective diffusivity

Heat and moisture, in their diffusion (i.e. conduction) through a porous medium such as a textile material, follow laws which are identical in their mathematical expression, and if the medium absorbs moisture the two processes are interlinked in a way which, mathematically, resembles the coupling of two vibrations. It would occupy too much space to give the theory here, but Henry (1939, 1948) has discussed

it at length with special reference to cotton as the medium, and some of the results will be quoted here. It is not expected that a reader who has not studied the papers referred to will be able to follow the details of the calculation given below, but it was thought worth while to give the various stages, using the symbols of the previous papers, so that anyone sufficiently interested could check the calculations or work out the effects of conditions different from those assumed here. Those not wishing to follow the calculation in detail should pass on to the paragraph preceding equation (8).

First, the conditions to be assumed must be decided on. Let it be supposed that the material is cotton having the characteristics described by Henry (1948), which were based upon experimental work by Urquhart & Williams (1924), Rees (1948) and others. These workers referred to cotton which was absorbing water rather than losing it, and this is in the right direction since the cotton will have absorbed water during the reversible heating up and will continue to gain moisture slightly during the irreversible stage. Hysteresis in the moisture relations of cotton will only become apparent when the final cooling stage starts, and will probably result in the final moisture content being 1–2% higher than the initial value.

The closeness of packing of the cotton will no doubt vary widely from drum to drum, but it was decided to take for calculation purposes a likely value (0·18 g/cm^3) for the overall density of several layers of huckaback towelling, partly for comparison with the experiments of Penikett *et al.* (1958) and partly because, as will be seen, it is really only the outside layer that is concerned, for the irreversible diffusive process is expected to get no further.

Assuming that the steam is at a gauge pressure of 20 lb/in.^{2} and that the atmospheric pressure is 760 mm Hg, the total pressure will be 760+1040=1800 mm Hg. This steam will be saturated at a temperature of 126°.

Two cases will be considered: that where the cotton starts dry ($M_\circ=0$), and that where the cotton originally contains 5% of moisture ($M_\circ=0{\cdot}05$), described by Penikett *et al.* (1958) as normal. We have already seen that the moisture contents during 'quasi-equilibrium' will be about 6% and 12% respectively. The relative humidity in equilibrium with the cotton which started dry, and has been superheated to 135°, is found from a table of vapour pressures to be 77%. That for the cotton starting at 5% will be higher and we may presume it to be 90%; the exact value will not appreciably affect the final conclusions.

In what follows, where two figures are given for any quantity, they refer to cotton starting dry and at 5% moisture content respectively, and the symbols other than those already introduced will be those used by Henry (1948). References to page numbers, figures, etc., in that paper will be denoted by FS.

From Fig. 4 of FS and the above values for the relative humidity it is estimated that σ will be about 100 and 500 cm^3/g. The effective density ρ_s of the cellulose (p. 249 of FS) will be 1/0·63 and 1/0·68, or 1·59 and 1·47 g/cm^3; hence γ (p. 254 of FS) is 0·20 and 0·21 g/cm^3. Thus $\gamma\sigma=20$ and 100 for the two cases; and since these are large compared with unity, we can assume that $1-\lambda\nu$ is equal to $1/(1+\varepsilon\omega)$ as explained near the bottom of p. 254 of FS. By estimation from Fig. 8 of FS we get $1-\lambda\nu=1/(1+\varepsilon\omega)=0{\cdot}12$ and 0·034, from which $\lambda\nu=0{\cdot}88$ and 0·97.

These values indicate 'strong coupling' between the diffusion processes for heat and moisture under the given conditions. Moreover, since moisture diffusion at the high temperature and in the relative absence of air will be much faster than is indicated by any of the values for D given in the Table on p. 256 of FS, while the heat diffusion will be little, if any, faster than corresponds to the values there given for $\boldsymbol{D}$, it is evident that the ratio $D/\boldsymbol{D}$ will be large compared with unity. Referring to the nomogram in Fig. 2 of FS, it is found that in these circumstances p, the relative amplitude of the secondary (fast) wave, is small, and can be neglected. Similarly, use of the nomogram in Fig. 1 of FS, but substituting 1/u for u (where $u=D/\boldsymbol{D}$) because we want D_1 in terms of $\boldsymbol{D}$ instead of in terms of D, reveals that D_1, the proper diffusivity for the main wave, is practically identical numerically with $\boldsymbol{D}$, the 'constant-vapour-concentration' diffusivity for heat. Hence the statement made above that it is this diffusivity which controls the rate of the irreversible stage under the conditions which have been assumed.

To calculate $\boldsymbol{D}$ (see p. 256 of FS) the value of $\boldsymbol{D''}$, the 'no-absorption' diffusivity for heat is calculated. This is given by $k/c\,\rho$, where k is the thermal conductivity of the package, c its specific heat, and ρ its density. We can find k from the experimental data of Speakman & Chamberlain (1930) for loose cotton, which were plotted against density of packing in Fig. 7 of FS; c can be found from the expression on p. 251 of FS, giving 0·38 and 0·45 cal $deg^{-1}g^{-1}$; and ρ has been taken as 0·18 g/cm^3. These values give $\boldsymbol{D''}=1{\cdot}5\times10^{-3}$ and $1{\cdot}2\times10^{-3}$ cm^2/sec. Now $\boldsymbol{D}=\boldsymbol{D''}/(1+\varepsilon\omega)$, and it has already been found that $1/(1+\varepsilon\omega)$ is 0·12 and 0·034: hence $\boldsymbol{D}=1{\cdot}8\times10^{-4}$ and $0{\cdot}4\times10^{-4}$ cm^2/sec for cotton starting dry and at 5% moisture content respectively.

Ordinary diffusion theory can now be applied, using the above values for the diffusivities, to find how fast the extra (irreversible) moisture will penetrate into the surface of the package. The appropriate expression is to be found at an early point in any work on heat conduction (e.g. Carslaw & Jaeger, 1947), and ideally applies to a semi-infinite solid bounded by a plane surface; but if the body concerned has all its dimensions large compared with the depth of penetration of the disturbance its shape does not matter much, and the theory will apply to the early stages of penetration which concern us. If a body is initially at a uniform temperature (or moisture content) and at time t=0 a change V is brought about at the surface and thereafter maintained constant, the change v at a depth x below the surface after time t is given by

$$v = V\left\{1-\mathrm{erf}(x/2\sqrt{\boldsymbol{D}t})\right\} \qquad (8)$$

where erf( ) denotes the function variously known as the Error Integral or the Probability Integral, which is obtainable from published Tables (Comrie, 1955; Milne-Thomson & Comrie, 1931).

To find the times required for changes in temperature or moisture content of one-tenth and one-half of the change at the surface to penetrate to various depths, V/10 and V/2 are substituted for v in equation (8). This gives

$$t_{0\cdot1}=x^2/5{\cdot}4\boldsymbol{D} \quad \text{and} \quad t_{0\cdot5}=x^2/0{\cdot}92\boldsymbol{D} \qquad (9)$$

Inserting in these expressions the values for $\boldsymbol{D}$ obtained above, we get the practical data shown in Table 2. The reader may like to be reminded at this point that the 'change' there referred to is the irreversible passage from the 'quasi-equilibrium'

state to the final equilibrium state; i.e. a fall in temperature from the 'superheat' temperature to that of the steam, and a rise in moisture content from the values given in Table 1 to something round about 20%. Table 2 gives the times required for one-tenth and one-half of this total change to penetrate to various distances from the surface of the package. The same slow rate of penetration would apply also to any drying out and heating up that occurred during the initial evacuation. Hence such effects are expected to be only superficial.

Table 2. *The rate of irreversible penetration of moisture into a cotton package after 'quasi-equilibrium'**

Depth of penetration		Time (min) for change to 10% and 50% of the surface change, with cotton starting			
		Dry		At 5% moisture	
cm	in.	10%	50%	10%	50%
0·25	0·1	1	7	5	30
0·5	0·2	5	25	20	120
1·0	0·4	20	100	75	450
2·5	1·0	100	600	500	3000

* With total surface changes from the conditions of Table 1 to about 20% moisture content.

Now it was found that two thicknesses of huckaback towelling under light pressure made a layer about 0·1 in. thick. Thus, if the above calculations prove to be valid, an outer wrapping of a very few thicknesses of such material should prevent the irreversibly absorbed moisture from penetrating to materials inside, under practical conditions of sterilization, with a consequent economy in drying time. To adapt the above results for different densities of packing (of the surface layer), and for different thicknesses, it may be assumed that the times are proportional to the density of the layer, and to the square of its thickness.

It will be noticed that no reference has yet been made to the metal drum; and in fact the calculations apply strictly to a bare package of cotton materials. If a good vacuum and dry steam have been used, however, the presence of the drum is not likely to affect the slow irreversible stage greatly, and any effect it had would be to slow it down still further, since the polished metal surface would restrict the rate of loss of heat by radiation. We can take the times given in Table 2, therefore, as minima if a drum is used.

Weakening of Cotton by Heating

Cotton subjected to a high temperature in the presence of air, especially if moist, suffers a slow diminution in strength additional to any that may have been produced by the use of oxidizing agents during bleaching or laundering. This weakening by heat ('heat tendering') usually results mainly from oxidation by the air, but if any traces of acid have been left in the cotton another type of tendering, not requiring the presence of oxygen, may accompany it. Materials that have been laundered are

more likely to have been left alkaline than acid, and articles such as gowns and towels that are used and laundered many times are not likely to suffer from acid-tendering. Evidence is lacking to show how far steam alone can damage properly prepared cotton, but it is certain that cotton which has been overbleached in manufacture or at the laundry is further damaged much more quickly than normal cotton when heated either in air or in steam. The damage in this case is accompanied by rapid discoloration to a yellowish or greyish brown colour, according to whether the heating has been in air or steam, respectively. The same is true of cotton that has been left acid through insufficient washing after the 'souring' which is the last stage of bleaching, or in other ways. This, however, is less likely to be met in practice than overbleaching, which may occur with surgical cotton goods owing to over-anxiety on the manufacturer's part to produce a very white absorbent product. It may also occur at the laundry. It seems likely that overbleaching, rather than any particular sterilization procedure, has been the prime cause of the 'charring' and weakening of dressings which various reports show may follow a relatively small number of sterilizations. Cotton that has been properly treated should stand a great number of steam sterilizations without becoming unserviceable (see e.g. Walter, 1948, Fig. 38).

Data have been published for the rate of tendering of properly bleached cotton heated dry in air. Since these are not directly relevant to the problem of sterilization in steam, it will suffice to summarize them by saying that bleached cotton heated in air at 120° (248°F) loses half its strength after about 300 hr, and every further 10° (18°F) above this temperature roughly halves the time. Heating in the presence of steam as well as air accelerates the oxidative tendering greatly, but published data do not say whether steam alone under the pressures used in sterilization can appreciably damage properly bleached cotton. With overbleached cotton it is believed that part, at least, of the damage wrought by steam can be regarded as development of 'latent damage' already done by the bleaching agent, and that the first steam treatment after the overbleaching will have more effect than any subsequent treatment. (Alkaline conditions have the same 'developing' effect on the 'latent damage'.) On the other hand, the effect of heating acid cotton, whether in air or steam, is certainly progressive, each successive sterilization doing further damage.

The immediate cause of the loss of strength in all the cases mentioned above is thought to be a reduction in the average length of the cellulose chain molecules, which are severed at various points by the chemical attack. Several methods of estimating the average length are available, and one in particular, the 'fluidity test', is in common use in the textile industry for estimating the extent of chemical attack separately from the various other factors that influence strength. It is a useful method of detecting overbleaching and hence the liability of cotton goods to excessive deterioration on heating. A description of the method of carrying out the test is given by the British Standards Institution (Specification, 1955).

The Advantages of a Good Vacuum

The advantages to be gained from drawing a good vacuum (say, one in which not more than 3% of the air remains behind) before the admission of steam have been

well demonstrated experimentally by Knox & Penikett (1958), as well as the desirability of using dry steam, by Penikett, Rowe & Robson (1958). It may be useful, however, to give a brief account of the advantages of a good vacuum from the standpoint of the considerations given in this paper.

In the absence of a good vacuum there is certain to be air remaining for a long time inside the drums, especially in the interstices of the cotton materials (Alder & Gillespie, 1957). Little reliance can be placed on sweeping out the air by blowing off steam when it is first admitted. Air may be effectively displaced from the autoclave itself, but it is difficult to see how any sweeping action at all could take place inside the drums. Any steam that reaches the interior of the package to heat it up by absorption must have got there by gravity displacement of, or diffusion through, the air. Rough calculations based on measured values of the air permeability of textiles, and on the difference in density of the air inside and the steam outside, show that it is likely to take as much as 1 hr for the air to be displaced by gravity from an average drum full of cotton materials, and diffusion would be even slower, particularly if the ventilation holes in the drum are small. Exit of air from the drum will also be hindered by the inward flow of steam through the apertures as it is absorbed. Air may even be carried into the drum by the entering steam: this concentration of air just where it is not wanted whenever steam is condensed or absorbed is all too familiar to chemical engineers, who find that a quite low original percentage of air in steam may accumulate at a condensing surface and thus greatly retard the transfer of heat from steam heating coils. Heat will, to a slight extent, be conducted in from outside without passage of steam, but we have seen that this process (or rather its inverse) is so slow that it can be neglected except for the outside layers. Thus while air is still in the drum all ways of heat transfer are slow, and the centre will not reach sterilizing temperature till the outside has been hot for some time (Knox & Penikett, 1958; Walter, 1948, Fig. 57; Savage, 1937). The heating of the centre could be hastened by raising the outside to an otherwise unnecessarily high temperature, but only at the cost of increasing the tendering rate. What is perhaps worse than the slow and non-uniform rate of heating up is the fact that it will vary from drum to drum according to the nature of the package inside, and the amount of air left in it, so that to be sure of sterilizing all drums right to the centre a time long enough for the slowest case must be allowed (and even then it is not unknown for a drum's contents to be rejected because the indicator has not turned colour).

With a good vacuum there is nothing to impede the passage of steam through the interstices except its own viscosity and inertia, and the heating is uniform, rapid and reversible, the temperature coming up to a definite predictable point in as little as 1 min (Knox & Penikett, 1958) and staying there. Further, on removal of the steam the cotton will be nearly as dry as when it was put in, immediately it has come down to room temperature (Penikett, Rowe & Robson, 1958). The steam ejector type of vacuum pump is not usually effective enough; those that the author has seen produced vacua which were quite insufficient.

The advantages of a good vacuum, therefore, are: (1) uniformity of heating throughout the drum; (2) uniformity of heating between one drum and another, with less dependence upon skill in the packing; (3) regularity of the heating-up time

from one day to another; (4) certainty that the maximum temperature will be, within a degree or two, that of the steam; (5) rapidity of heating up, though the time thus saved will be partially offset by the time required for evacuation, which will depend upon how much money can be spent on the pump; (6) reversibility of the heating and of the absorption of moisture, so that no long drying period is needed, though this is also dependent upon the use of dry steam; (7) less heat-tendering, owing to the shorter duration of heating, which will not require to exceed greatly the time needed to destroy bacteria, etc.

It has been suggested (e.g. Walter, 1948) that the use of a good vacuum would give rise to such superheating that the effectiveness of sterilization would be diminished and the life of the materials seriously shortened. Reasons have been given above for supposing that such effects would be no worse than those encountered when the gravity displacement method is used. The author can see no disadvantage pertaining to the good vacuum other than that of the increased first cost of the apparatus. This may well in practice be offset by the greater amount of work that a sterilizer of given size could perform in a day.

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THE JOURNAL OF APPLIED BACTERIOLOGY

CONTENTS, VOL. 22, No. 1

PAGE

The Effect of Nisin on the Growth of Cells and Spores of *Clostridium welchii* in Gelatine. By J. E. Eastoe and Joan E. Long 1

The Inactivation of *Bacillus Subtilis* Penicillin by Gamma Spores in Radiation. By E. C. Hill and G. O. Phillips 8

Mycological Synthesis of Fat from Whey. I. Preliminary Studies with Stationary Cultures. By P. Wix and M. Woodbine 14

A Study of a Gram-Positive Coccus Responsible for Ropiness and Viscosity in Malt Wort. By J. W. Hopton and A. N. Hall 23

Coli-Aerogenes Bacteria in Farm Water Supplies. By S. B. Thomas, Phyllis M. Hobson and R. G. Druce 32

The Bacterial Content of Farm Dairy Equipment. By D. G. Griffiths and S. B. Thomas 46

The Microbiological Examination of Butter. By R. G. Druce and S. B. Thomas . 52

Cold-Tolerant Fermentative Gram-Negative Organisms from Meat and Other Sources. By B. P. Eddy and A. G. Kitchell 57

Some Observations on Bactericidal Paint Films. By E. J. Morris and H. M. Darlow 64

The Sanitation of Fish Boxes. I. The Quantitative and Qualitative Bacteriology of Commercial Wooden Fish Boxes. By R. Spencer 73

A Study of the Bacteria Associated with the Roots of Subterranean Clover and Wimmera Rye-Grass. By Joan I. Sperber and A. D. Rovira 85

The Influence of Environmental Factors on the Microbicidal Effect of Ionising Radiations. By B. A. Bridges and T. Horne 96

Observations on the Isolation of Salmonellae from Selenite Broth. By H. G. Smith 116

The Effect of Freezing and Storage on the Bacterial Flora of Pasteurized Milk. By J. G. Murray and W. E. Coey 125

The Occurrence of *Bacillus cereus* in Milk and on Dairy Equipment. By Kathleen O. Donovan 131

The Investment Value of Continuous Culture. By R. Elsworth, R. C. Telling and D. N. East 138

The Value of the Association Escherichieae-Group D Streptococci in the Diagnosis of Contamination in Foods. By R. Buttiaux 153

Physical Aspects of Sterilizing Cotton Articles by Steam. By P. S. H. Henry . 159

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